TROPANE ALKALOID PRODUCTION IN IMMOBILIZED PLANT CELL CULTURES

by

MARGARET ANN COLLINGE BSc.

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DECLARATION

I hereby declare that this thesis was
composed by myself, and the work described
herein to be my own.

Margaret A. Collinge
Edinburgh, 1987.

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Margaret A. Collinge. 1987.

ABBREVIATIONS

cat. no.	catalogue number
conc	concentration
°C	degrees centigrade
c.	*circa*
cf.	*confer*
CPA	para-chloro-phenoxyacetic acid
2,4-D	2,4-dichloro-phenoxyacetic acid
et al	*et alia*
f. wt.	fresh weight
λmax	the wavelength at maximum absorption
g	gramme(s)
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Cromatography
i-N	inorganic nitrogen
i-P	inorganic phosphate
l	litre(s)
L-orn	L-ornithine
L-phe	L-phenylalanine
K	kinetin
m	metre(s)
μ (as prefix)	micro (10^{-6})
m (as prefix)	milli (10^{-3})
MeCN	acetonitrile
MeOH	methanol
min	minute(s)
mol	mole(s)
M	molar (1 mole/litre)
MS	Mass Spectrometry
MS medium	Murashige and Skoog medium
MS-CDK	MS medium + 2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, *0·1* mgl^{-1} K
MS-NK	MS medium + 1.0 mgl^{-1} NAA, and 0.1 mgl^{-1} K
n (as prefix)	nano (10^{-9})
NAA	naphthaleneacetic acid

Na-ppyr	Na-phenylpyruvate
ND	not detectable
no.	number
pH	negative log of the hydrogen ion concentration
ppi	pores per inch (in polyurethane foam)
psi	pounds per square inch
rpm	revolutions per minute
s.e.	standard error
SH	Schenk and Hildebrandt medium
spp	species (plural)
SSM	Standard Synthetic Medium
TLC	Thin Layer Chromatography
u.v.	ultra violet
v/v	volume per unit volume as percentage
w/v	weight per unit volume (gl^{-1}) as percentage
wt.	weight
xg	times the force of gravity
$\bar{x}$	mean of values of x
$\bar{y}$	mean of values of y
>	greater than
<	less than
%	percent

CONTENTS

ABSTRACT

The aims of this project were to investigate the control of tropane alkaloid production in plant cell cultures; and to find ways of increasing the yield of the anticholinergic drug atropine (dl-hyoscyamine) which would be compatible with a system for its commercial production by cultured cells.

The cultures used were derived from *Atropa belladonna* and *Hyoscyamus muticus*, which are common commercial sources of atropine. Their tropane alkaloid content was determined using an extraction procedure and an HPLC system developed for this project. The cells were immobilized in order to promote the slow growth and high degree of aggregation which are generally associated with increased secondary metabolite production. An immobilization method, using polyurethane foam as the inert matrix, was developed and characterized for cells of *A. belladonna* and *H. muticus*. There is an indication that immobilized cells produced more atropine than suspended cells. The investigation of the control of tropane alkaloid production *in vitro* was carried out using cultures of *H. muticus* because they produced more alkaloid than those of *A. belladonna*.

Certain alkaloid precursors when added to the culture medium increased the yield of atropine, which suggests that there may be competition between primary and secondary metabolic pathways for amino acid precursors. However, inhibiting culture growth, and thus primary metabolism, by limitation of nitrogen and/or phosphate in the culture medium, did not induce alkaloid production when the control cultures produced less than detectable levels of alkaloids.

Particular attention was given to an examination of the proposed link between structural differentiation and tropane alkaloid production, but no relationship was found between the number of roots formed in a culture and its atropine content. However, some culture lines derived from root bearing aggregates produced higher levels of atropine than those derived from non-root bearing aggregates. The possible reasons for this and the relative importance of the selection of high producing lines, and manipulation of the culture medium are discussed.

The proposed systems for the production of valuable secondary metabolites by immobilized plant cell cultures require that they release the products into the culture medium. Since the cultures of *H. muticus* used in this study accumulated atropine intracellularly, attempts were made, using physical treatments and a model system of anthocyanin release, to find a permeabilization method for these cultures. Treatment with low external pH was identified as a method which, with further development, could be used to permeabilize cells of *H. muticus*, without causing loss of their viability or their ability to produce more alkaloids.

CHAPTER ONE

INTRODUCTION

Plant secondary metabolism is studied for three major reasons: i) an intrinsic interest in secondary metabolic pathways; ii) the synthesis and accumulation of secondary products can be seen as a facet of cytodifferentiation, so the expression of secondary pathways can be useful markers in studies of the control of differentiation; iii) many secondary products are valuable to man for a wide variety of applications: as pharmaceuticals e.g. codeine, digitoxin, atropine; as food additives e.g. capsaicin, saffron; for crop protection e.g. pyrethrin; and as perfume ingredients e.g. aromatic oils. For many years attempts have been made to produce such compounds from tissue cultures in order to obtain a qualitatively and quantitatively consistent supply at reduced cost.

The research project described in this thesis was concerned with the production of tropane alkaloids *in vitro*. In this chapter the rationale of the approach will be described and justified. The first major section in the chapter deals with the nature and use of tropane alkaloids, the sources from which they are derived and their production in the plant. The second sets out the strategy for the project and its derivation from the findings of previous research on secondary metabolism in cell cultures.

TROPANE ALKALOIDS - THEIR VALUE AND PRODUCTION

Tropane alkaloids are esters of organic acids with heterobicyclic amines. They are produced by members of the Convolvulaceae, Dioscoraceae, Erythroxylaceae, and Solonaceae (Holmes 1950). The most widely known tropane alkaloid is l-hyoscyamine, which is an ester of tropine and tropic acid. During its extraction from plants racemization often takes place, forming dl-hyoscyamine which is also known as atropine. The other alkaloids which are present in appreciable amounts in the Solanaceae are scopolamine (dl-hyoscine) and hyoscyamine-N-oxide (Fig 1.1).

The Uses of Tropane Alkaloids

Tropane alkaloids compete with acetylcholine for postganglionic cholinergic receptor sites in the autonomic nervous system. This accounts for the toxicity to animals of the plants which contain them, and for their value as pharmaceuticals (Lewis & Elwin-Lewis 1977). They are used in the treatment of a wide variety of illnesses, e.g. as opthalmics, antispasmodics and antianginals, in the treatment of Parkinson's disease, as a preoperative medication, and as an antidote to certain chemical weapons.

The Current Commercial Sources

Since synthetic production of these alkaloids is difficult and costly, they are derived from the dried tissues of various members of the Solanaceae. *Atropa belladonna* (Deadly Nightshade) which is cultivated in Southern Asia, China, and North America is perhaps the most well known source of atropine, but its major sources are wild *Hyoscyamus muticus* (Egyptian Henbane) from Egypt, the Sudan, and the United Arab Republic, and *Duboisia leichhardtii* and *D. myoporoides* from Australia (Sarin 1982, Cordell 1981).

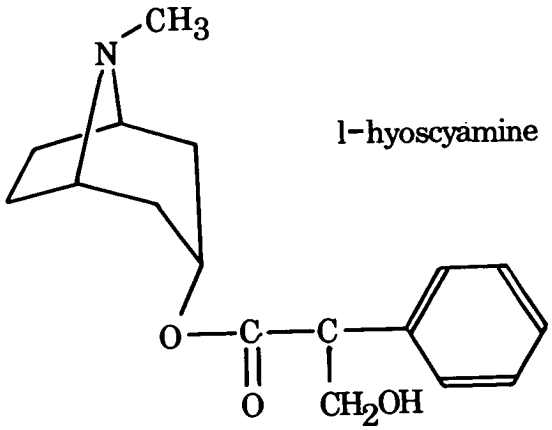
In India the large scale cultivation of both indigenous and exotic species failed, and the reasons were considered to be the high cost of cultivated material, the frequent incidence of viral and fungal diseases, the variability in alkaloid content, and the limited availability of suitable land and labour (Sarin 1982). Add to these problems the variable political, economic, and climatic environment in the areas from which the plants are exported, and there is a clear case for attempting to produce atropine, and the other much used tropane alkaloid, scopolamine, from cultured cells rather than from whole plants. Furthermore,

the wide variety of applications of these alkaloids means that although their use in some cases may be superseded by other drugs, they are unlikely to become redundant, at least in the near future.

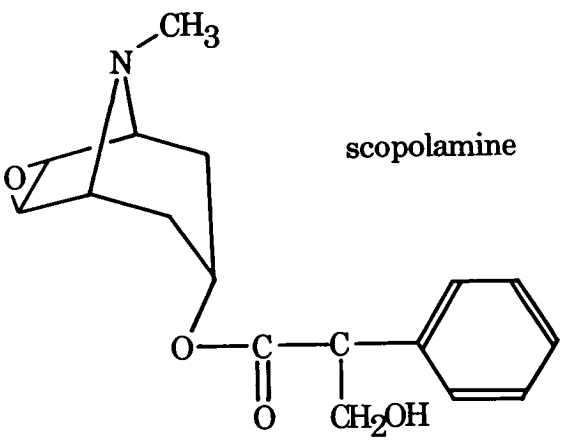
In this study the most commonly used wild and cultivated sources of atropine, *Hyoscyamus muticus* and *Atropa belladonna* respectively, have been compared for their ability to produce alkaloids *in vitro*.

The amount of information available about the alkaloid content of *H. muticus* is limited. Members of the genus *Hyoscyamus* normally contain hyoscyamine, scopolamine, and their N-oxides; although the presence of apohyoscyamine, norhyoscyamine, littorine, tropine, Ψ tropine, and cuscohygrine has been recorded (Evans 1979). In *H. muticus* 90 % of the total alkaloid present is hyoscyamine, and analyses have found 0.5-0.6 % dry weight hyoscyamine in stems, 1.4-1.7 % in leaves and 2.0 % in flowers (Morton 1977). The minimum content permissible by the British Pharmacopoeia is 0.5 % dry weight (Merck Index 1983).

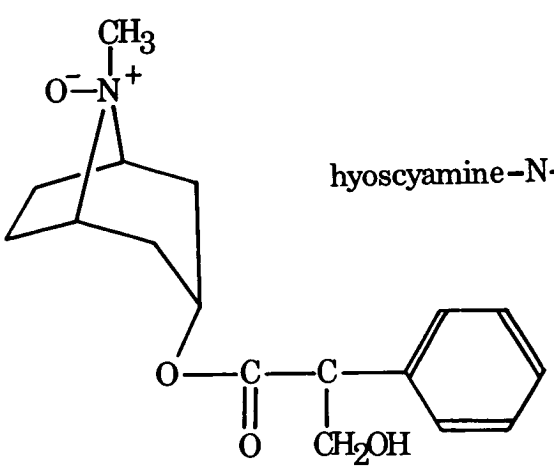
The roots and leaves of *A. belladonna* are harvested from cultivated plants, and must contain a minimum of 0.4, and 0.3 % dry weight of total alkaloid (respectively) for commercial purposes (Merck Index 1983), although the levels can reach 0.7 and 0.6 % total alkaloids in roots and leaves respectively (Morton 1977). The highest alkaloid contents are found in the inflorescences and fruits, up to 0.2 % fresh weight of total alkaloid (Phillipson & Handa 1976). The alkaloids present in the leaves are l-hyoscyamine, scopolamine and their N-oxides, 3α-phenylacetoxy-tropane, apoatropine, aposcopolamine, norhyoscyamine, 6-hydroxyapoatropine, and 6-hydroxyhyoscyamine; in addition to these the roots contain small amounts of belladonnine (an apoatropine dimer), hygrine, and cuscohygrine (Phillipson & Handa 1975, Evans 1979, Oprach *et al* 1986).



l-hyoscyamine



scopolamine



hyoscyamine-N-oxide

Fig. 1.1

Diagrams showing the structures of the major tropane alkaloids produced by members of the Solanaceae; l-hyoscyamine, scopolamine, and hyoscyamine-N-oxide.

The Distribution of Alkaloid in the Plant

Both the total amount of alkaloid and the proportion of the different alkaloids varies with the stage of vegetative development, and between the different parts of the plant. In *A. belladonna* atropine is always the predominant alkaloid, accounting for 70-85 % of the total tropane alkaloid content (Phillipson & Handa 1976). The scopolamine content is highest in the young plant, decreasing quite rapidly with age. Also, at any one stage of the growth period, the young leaves at the top of the plant contain the highest alkaloid levels and are rich in scopolamine, while the older leaves contain less alkaloid and a smaller proportion of scopolamine (Wilms *et al* 1977). Hyoscyamine-N-oxide usually makes up < 10 % of the total alkaloid, but in inflorescences it reaches 12 % and in fruits 20 % (Phillipson & Handa 1976). The maximum amount in the whole plant is found at the stage of ripening fruits (Wilms *et al* 1977). But in each organ or tissue, the highest concentrations are always reached when it is actively growing.

Although they are found in all parts of the plant, the alkaloids are concentrated in specific cells or tissues. In the aerial parts they are mostly found in the epidermal cells and in the phloem and xylem parenchyma. In roots they accumulate in the root cap, and in the outer cortex and phloem parenchyma of older roots (Cromwell 1955).

Evidence for their accumulation being specifically controlled comes from studies with grafts between *A. belladonna* and tomato plants (which do not produce tropane alkaloids). Tomato scions grafted onto *A. belladonna* root stocks accumulated alkaloid in the blade of the leaf, but in the aerial parts of *A. belladonna* the highest alkaloid content was found in the petiole and midrib (Mothes 1955). In addition to the changes in the amounts of alkaloid present in each organ during the vegetative period, large fluctuations in the alkaloid content of leaves and fruits during the day were reported by Fairbain & Wassel (1967). This indicates that alkaloids are not slowly accumulated waste products, but rather that they are rapidly turned over and actively accumulated by certain cells.

The Site of Alkaloid Formation

Although the tropane alkaloids are found in all parts of the Solanaceae that contain them, the available evidence is consistent with their synthesis taking place chiefly in the roots followed by their transport to the aerial parts. This evidence is derived from grafting experiments performed by Romeike (reviewed in Mothes 1955) and Warren-Wilson (1952) between species which form tropane alkaloids and those which do not. The aerial parts (scion) of, for example, *A. belladonna* grafted onto the roots (stock) of, for example, tomato are virtually alkaloid free. In the reverse graft, i.e. tomato scion on *A. belladonna* stock, the scion tissue contains appreciable or high levels of alkaloids. Roots growing from alkaloid free *A. belladonna* scions still attached to a tomato stock contained alkaloids at concentrations only slightly lower than those in normal *A. belladonna* roots (Warren-Wilson 1952). However, the aerial parts of alkaloid containing species do not appear to be completely incapable of producing alkaloids. When *Atropa* scions on tomato stocks were cut down to very small shoots, thus removing almost all preformed alkaloids, then grown on, they still accumulated alkaloids (Mothes 1955). Also detached leaves of *A. belladonna* are able to produce alkaloids (James 1949).

Transport of the alkaloids probably takes place chiefly in the xylem, and alkaloids have been found in bleeding sap (Waller & Nowacki 1978), but its downward transport, probably in the phloem, has also been established by Warren-Wilson (1952).

The role of roots at least as the chief site of tropane alkaloid synthesis *in vivo* has led to the suggestion that root formation may be required for, or could increase alkaloid production *in vitro* and this has been investigated in this study.

The Biosynthesis of Tropane Alkaloids

The Biosynthesis of Hyoscyamine

Hyoscyamine is produced by the esterification of tropine and tropic acid. Its synthesis has been reviewed by Leete (1979) and Waller & Dermer (1981), and is shown in Fig 1.2.

Synthesis of the Tropine Moiety

Acetic acid, probably in the form of acetoacetate or acetoacetyl coenzyme-A, and

L-ornithine (L-orn) combine to form tropine as shown in Fig 1.2. Leete (1979) considers that the synthesis proceeds via δ-N-methylornithine rather than putrescine, despite the incorporation of added putrescine into hyoscyamine, because incorporation of [2-^{14}C]orn yielded tropine labelled only at the C1 bridgehead carbon. If putrescine, which is a symmetrical molecule, was a free intermediate, labelling at both the C1 and the C5 positions would be expected. However, δ-N-methylornithine has not been detected *in vivo*; and plants of *A. belladonna* and *Datura innoxia* contain relatively high levels of ornithine decarboxylase, putrescine N-methyl transferase and N-methyl putrescine oxidase (Waller & Dermer 1981, Leete 1979). Alternative routes from L-orn to hygrine have been proposed (Waller & Dermer 1981, Torsell 1983) but N-methyl putrescine is an established precursor of tropine, and 4-methyl-aminobutyraldehyde has been detected in *Datura* plants fed with [2-^{14}C]ornithine (Leete 1979). Hygrine is a known intermediate of tropine synthesis, and is also the immediate precursor of cuscohygrine, a hygrine dimer. The route from hygrine to tropine is hypothetical, although tropinone has been detected in *Cyphomandra* species (Leete 1979) and recently in *A. belladonna* (Oprach *et al* 1986); and alternative pathways via hygroline have been ruled out (McGaw & Wooley 1983).

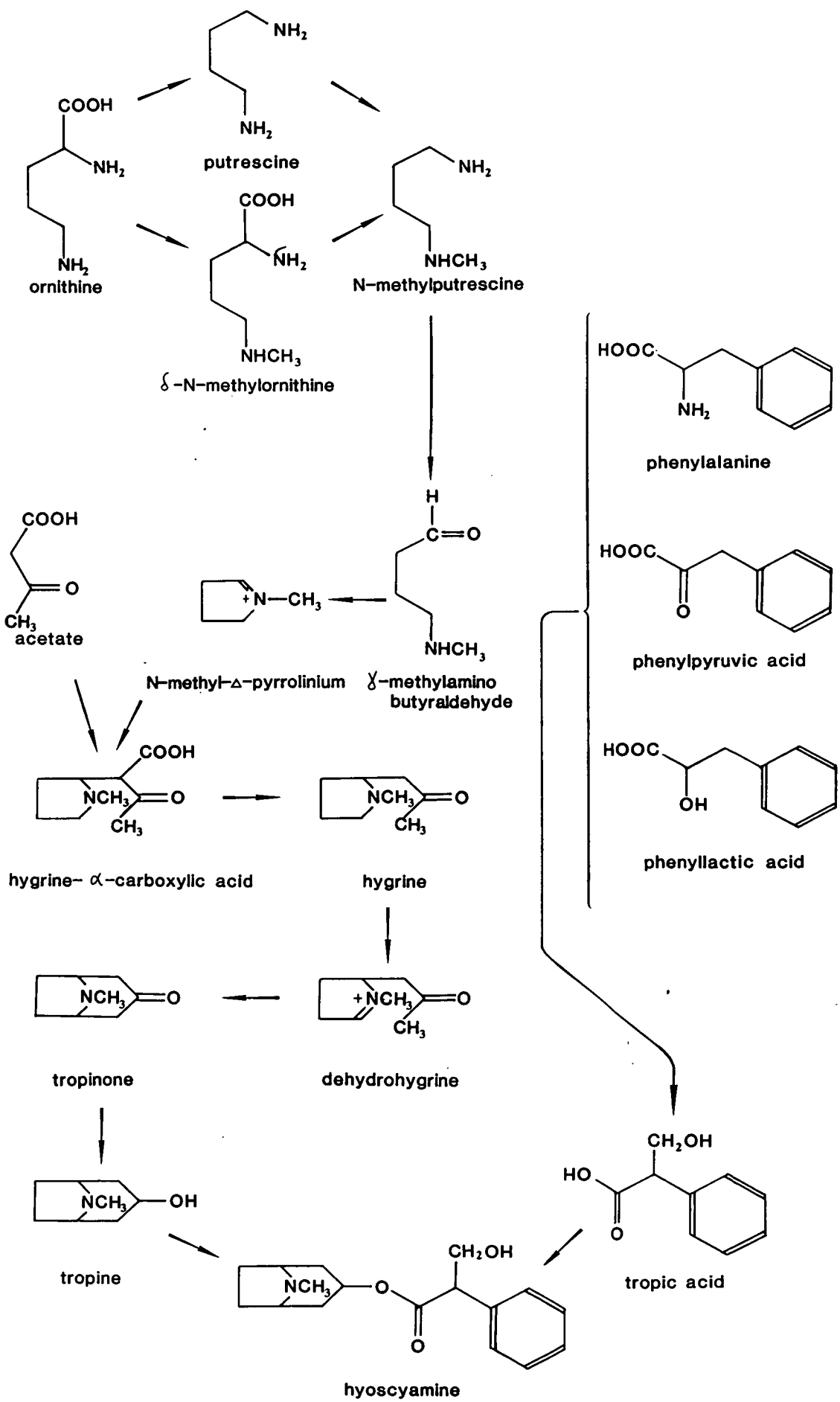
The Synthesis of Tropic Acid

Tropic acid is derived from L-phe by an intramolecular rearrangement of the side chain (Leete *et al* 1975). Phenylpyruvic acid and phenyllactic acid have also been shown to be precursors of tropic acid, but it is not known whether they are incorporated via L-phe, or are intermediates in the pathway (Evans & Wooley 1976). A hypothetical scheme for the intramolecular rearrangement has been devised by Dalton (1979), in which the final intermediate is αformylphenylacetic acid. This substance was found not to be incorporated into tropic acid by Leete (1979). However it is a known constituent of *A. belladonna* and *Datura innoxia* plants, and its pronounced instability may account for its failure to act as a precursor (Gross *et al* 1981)..

The Formation of the Minor Alkaloids

There is little information available about the synthesis of the minor alkaloids of *A. belladonna*, but they are generally thought to be derived from hyoscyamine. Phillipson & Handa (1976) considered it more likely that hyoscyamine-N-oxide is formed by oxidation of hyoscyamine, than by oxidation of tropine followed by esterification with tropic acid. At least some of this oxidation takes place in the fruits of *A. belladonna*.

The conversion of hyoscyamine to scopolamine proceeds via 6β-hydroxyhyoscyamine (Hashimoto & Yamada 1986) and probably 6,7-dehydroxyhyoscyamine although the latter has yet to be isolated from plant material.


putrescine
ornithine
δ-N-methylornithine
N-methylputrescine
acetate
N-methyl-Δ-pyrrolinium
γ-methylamino butyraldehyde
phenylalanine
phenylpyruvic acid
phenyllactic acid
hygrine-α-carboxylic acid
hygrine
tropinone
dehydrohygrine
tropine
tropic acid
hyoscyamine

THE PRODUCTION OF SECONDARY METABOLITES BY PLANT CELL CULTURES

The advantage of studying secondary metabolism *in vitro* is that the cells are removed from the complex environment of the plant, and instead are placed in a controllable, defined environment which allows the effect of individual parameters to be examined. The advantages of *in vitro* production of useful compounds for commercial use are: freedom from climatic restrictions, limitation of suitable land and labour, and from crops and diseases; together with the assurance of a qualitatively and quantitatively consistent product. As described above (Sarin 1982) these general commercial considerations also apply to the specific case of atropine production.

In this section the findings of previous research into tropane alkaloid production by plant cell cultures are described, in order to justify the study presented in this thesis (Chapter Three).

Previous Results

Generally plant cell cultures which are friable and fast growing seldom accumulate secondary metabolites at levels which occur in the intact plant. There is good evidence that their production, often with increased yield, is related to a slow or zero growth rate, aggregation, and/or morphogenesis to form recognisable plant organs or embryos. This evidence has been reviewed by Yeoman *et al* (1980), and Yeoman *et al* (1982) and is therefore not described here in detail except for the specific cases of tropane alkaloid production *in vitro*, which are summarized in Table 1.1.

The effect of slow growth on product yield has been demonstrated in root cultures of *H. albus* (Hashimoto *et al* 1986); and in callus cultures of *A. belladonna* (Khanna *et al* 1977, and Sharma & Khanna 1982) where the maximum atropine content was reached six weeks after the cells were transferred to fresh medium. Also suspension cultured cells of *Datura innoxia* in the linear phase of growth contained less tropane alkaloids than those in stationary phase (Lindsey & Yeoman 1983a). In the latter publication it was also shown that alkaloid yields in various species were greater in those callus or suspension cultures with one or more of the following characteristics; slow growth, a high degree of aggregation, green colouration.

Table 1.1 Tropane alkaloid production *in vitro* by various members of the Solanaceae.

Description of the culture conditions has been limited to the culture type, the growth medium, and the growth substances added to it, although the experimental conditions will have varied widely. The total alkaloid and atropine contents have been given as percentage dry weight. This value has been determined in a number of different ways, including the use of Dragendorff's reagent, the Vitali Morin test, TLC, HPLC, and GLC. Furthermore, in some cases the value for total alkaloids includes all the tropane alkaloids, in others, only the major alkaloids (atropine, scopolamine and their N-oxides). This is not a complete list of all the previous work on this subject, and the experimental conditions and methods vary, but an indication is still given of the comparative ability of organized and unorganized cell cultures to produce tropane alkaloids.

Species	Culture Type	Basal Medium
Atropa belladonna	root callus	Guatheret's + supplements
"	cultured roots	Gautheret's + supplements
"	callus	Murashige & Skoog
"	callus	Murashige & Skoog
"	callus	Murashige & Skoog
"	callus	Murashige & Skoog
"	cultured roots	Street & McGregor
"	callus	Murashige & Skoog
"	cultured roots	Linsmaier & Skoog
"	cultured roots	Murashige & Skoog
"	transformed roots	Murashige & Skoog
Datura innoxia	suspended cells	Linsmaier & Skoog
"	callus	Linsmaier & Skoog
"	callus	Murashige & Skoog
"	callus	Murashige & Skoog
"	cultured roots	Linsmaier & Skoog
Datura strammonium	cultured roots	Linsmaier & Skoog
"	transformed roots	Gamborg's B5
Duboisia leichardtii	cultured roots	Gamborg's B5
"	cultured roots	Linsmaier & Skoog
Hyoscyamus muticus	callus	Murashige & Skoog
"	normal and transformed roots	Murashige & Skoog
"	cultured roots	Linsmaier & Skoog
Hyoscyamus niger	suspended cells	Schenk & Hildebrandt
"	H15 suspended cells	Linsmaier & Skoog
"	H15 cultured roots	Linsmaier & Skoog
"	cultured roots	Linsmaier & Skoog
Scopolia parviflora	callus	Linsmaier & Skoog
"	S22 transformed roots	Knop's

Growth Substances (M)	Alkaloid Content (% dry weight)		Reference
	Atropine	Total	
0	0.05	-	West & Mika (1957)
coconut milk	0.042	-	"
$5x10^{-6}$ 2,4-D	0.53	-	Khanna *et al* (1977)
$5x10^{-6}$ 2,4-D	0.5	-	Sharma & Khanna (1982)
$5x10^{-6}$ K	0.75	-	"
$4x10^{-6}$ BA	-	$1.1x10^{-3}$	Eapen *et al* (1978a)
0	0.26-0.45	-	Mitra (1972)
10^{-5} 2,4-D, 10^{-5} K	-	0.1-0.5	Lindsey & Yeoman (1983a)
0	-	0.35	Hashimoto & Yamada (1986)
0	0.031	-	Kamada *et al* (1986)
0	0.371	-	"
10^{-6} 2,4-D	-	$4.3x10^{-3}$	Hiraoka *et al* (1973)
10^{-6} 2,4-D	-	0.02	Hiraoka & Tabata (1974)
$2x10^{-5}$ 2,4-D, $5x10^{-5}$ K	-	0.334	Szoke *et al* (1982)
10^{-5} 2,4-D	-	1.0	Lindsey & Yeoman (1983a)
0	-	0.469	Hashimoto & Yamada (1986)
0	-	0.423	"
0	0.3-0.6	-	Payne *et al* (1987)
10^{-5} IBA	0.53	-	Endo & Yamada (1985)
0	-	0.359	Hashimoto & Yamada (1986)
$5x10^{-7}$ NAA, $5x10^{-6}$ K	-	0	Flores & Filner (1985)
0	-	0.5	"
0	-	0.248	Hashimoto & Yamada (1986)
10^{-5} 2,4-D	-	0.057	Dhoot & Henshaw (1977)
10^{-5} NAA, 10^{-6} BA	0.05	0.054	Hashimoto & Yamada (1983)
10^{-5} NAA, 10^{-6} BA	0.06	0.27	"
0	-	0.17	Hashimoto & Yamada (1986)
10^{-6} 2,4-D, 10^{-6} K	-	0.009	Tabata *et al* (1972)
0	1.3	-	Mano *et al* (1986)

Although unorganized cultures have produced tropane alkaloids, RajBhandary *et al* (1969), Thomas & Street (1970), and Endo & Yamada (1985) showed that this was dependant on root formation in the cultures, and Lindsey & Yeoman (1983a) found greater levels of alkaloids in embryogenic cf. "undifferentiated" suspension cultures of *A. belladonna*.

Eapen *et al* (1978ab) recorded no difference in the alkaloid content of unorganized and shoot forming callus, but in the former, scopolamine was the predominant alkaloid, rather than atropine as in the shoot forming callus and in the intact plants. Similarly Dhoot & Henshaw (1977) found that the loss of the ability to form roots by cultures of *H. niger* was associated with loss of the ability to produce hyoscyamine, but not in a decrease in the total alkaloid content. Scopolamine production has been associated with root development. During regeneration of plants from cell suspension cultures of *Datura innoxia*, scopolamine was not observed until roots were formed on the developing shoots (Hiraoka & Tabata 1974), and root cultures derived from a suspension culture line of *H. niger* produced a similar amount of atropine but much greater amounts of scopolamine (Hashimoto & Yamada 1983).

The Growth Rate

The inverse relationship between growth rate and secondary metabolite production may be due to competition between primary and secondary metabolic pathways for energy sources and substrates. Tropane alkaloids are derived from the amino acids L-orn and L-phe which in actively growing cells are required for protein synthesis. Some evidence for this comes from experiments with detached leaves of *A. belladonna* placed in a sucrose solution (James 1949). Their alkaloid content increased after three days and this was associated with a loss of protein nitrogen and an increase in soluble nitrogen. Also linear and stationary phase cells of *D. innoxia* incorporated DL[-^{14}C]ornithine into protein and alkaloids differentially, a greater proportion of the label being found in alkaloids in the stationary phase cells (Lindsey & Yeoman 1983a).

Therefore in this study the effect on alkaloid production of growth inhibition, exerted by the limitation of nitrogen and phosphate, was examined; and the extent to which added precusors could relieve any limit on alkaloid yield was investigated.

The Aggregation of Cells

The higher yields from aggregated cultures are thought to be achieved because the environment of the cells within these aggregates is similar to that in the intact plant. Cells within the aggregates will be exposed to gradients of nutrients, growth substances, dissolved gases (O_2 and CO_2) and light, and will be subject to position and pressure differences. Thus callus cultures are often more productive than suspension cultures.

However, the environment of cells on solid medium is difficult to control, as only a small proportion of them are in contact with the medium, and callus cannot be grown easily on a large scale. Cells in suspension cultures are in close contact with the medium, and can be grown in large volumes (albeit with some difficulty) but product yield is often relatively low. Immobilization of cells can be seen as an intermediate between these two culture methods. Cells are either bound to a solid support or entrapped in an inert matrix, then grown in a shaken liquid medium, or placed in a supporting structure with medium passing over them. Immobilized cell cultures have been used in this study, the reasons for this and for choosing a particular immobilization method are given in the section entitled "The Immobilization of Plant Cells".

Morphogenesis in Cell Cultures

The need for organization into recognizable plant structures to increase or induce alkaloid production, and for the establishment of a normal alkaloid pattern was pursued in this study particularly since hyoscyamine synthesis *in vivo* seems to take place mainly in the roots. This can be justified by the recently increased interest, particularly by Yamada's group (see Table 1.1) in cultured roots rather than cell cultures: e.g. callus of *H. albus* and *H. niger* had a relatively low alkaloid content, and produced atropine as the major alkaloid, while root cultures formed more alkaloid most of which was scopolamine (Hashimoto & Yamada 1986).

A problem with root cultures is their slow growth rate compared with callus and suspension cultures, which would make the growth stage of a commercial production system very long and costly. The H15 cultured roots of Hashimoto & Yamada (1983) grew as fast as the cell suspension cultures from which they were derived, but they are probably an exception. A more promising alternative is to use roots which have been transformed by *Agrobacterium rhizogenes*, the agent of

"hairy root" disease. These transformed roots have the characteristics of rapid growth, stability and yields which are generally equivalent to those *in vivo* (e.g. Flores *et al* 1985, Kamada *et al* 1986). However, Mano *et al* (1986) showed that the establishment of high producing, rapidly growing transformed root lines is not always simple; as their clones of *Scopolia japonica* varied considerably in their growth rate, alkaloid content, and alkaloid pattern. However, they did isolate two clones one of which produced three times more scopolamine, and the other eight times more atropine than did the original roots.

The influence of organization on the alkaloid pattern indicated that it was necessary to monitor not just the alkaloid yield, but also to separate and determine the amounts of the most abundant tropane alkaloids, hyoscyamine, scopolamine, and hyoscyamine-N-oxide. Therefore a method to determine the major tropane alkaloids in plant cell culture extracts was developed for this study.

Procedures for the determination of tropane alkaloids include titrimetric, colorimetric, immunological, TLC, HPLC, GLC, and MS methods. The titrimetric methods lack specificity and sensitivity (Majlat 1982). The most widely used colorimetric method is the Vitali Morin reaction (Roberts & James 1947). It can be used to determine the total alkaloid content of a sample, or the amount of an individual alkaloid after separation by TLC. Individual alkaloids can be measured with great specificity and sensitivity by immunological methods (Lehtola *et al* 1982, Fankhauser *et al* 1986). Mixtures of tropane alkaloids, and extracts containing them, can be separated and determined with accuracy by HPLC (Brown & Sleeman 1978), and GLC (Wilms *et al* 1977). The most accurate identification of alkaloids can be attained by detecting with MS, e.g. the use of GLC/MS by Oprach *et al* (1986) to identify a wide range of alkaloids in extracts from tissue of *A. belladonna*. However, HPLC is becoming increasingly popular for the analysis of both pharmaceutical products (Brown & Sleeman 1978) and plant cell extracts (Plank & Wagner 1986, Mano *et al* 1986).

THE IMMOBILIZATION OF PLANT CELLS

The use of immobilized plant cell cultures for the production of secondary metabolites has been described and extensively reviewed (e.g. Brodelius *et al* 1979, Lindsey & Yeoman 1983b, Shuler *et al* 1983, Rhodes 1985, Lindsey & Yeoman 1986, Hall *et al* 1987) and will not be recounted in detail here. In the following two sections the advantages of cell immobilization and of the particular immobilization method chosen for this study are described.

The Advantages of the Immobilization of Plant Cells

The physiological reasons for the use of immobilized plant cells are: that they can be maintained in conditions promoting slow growth; they are highly aggregated, and the aggregate size can be controlled, they have increased stability; they are protected, e.g. from shear during mixing; and sequential treatments can be administered easily and without undue disturbance of the cells themselves.

The use of an immobilized culture system for commercial production presents a number of advantages in terms of bioreactor design. A simple design is possible, usually involving stationary supported cells over which medium passes from a separate reservoir. Thus there are no mixing problems i.e. of efficient mixing of the cells, damage due to shear stresses, or sufficient aeration (which can be performed in the medium reservoir). The medium can be renewed or changed completely without disturbing the cells, thus making microbial contamination less likely. The higher cell/medium ratio possible with this system is cheaper in itself, and also reduces the cost and difficulty of product recovery. As immobilized cells can be maintained for long periods of time, prolonged production phases in the bioreactor with continuous or intermittent product removal are envisaged. This reduces the relative length of the expensive, non-productive growth phase.

There are two possible disadvantages to an immobilized system i) the cost and the stability of the immobilization matrix, which is dealt with in the next section; ii) the necessity for the release of the product into the culture medium which is considered in the section headed "Product Release".

The Method of Immobilization

The methods of plant cell immobilization have not been described in detail here as they have been reviewed by Lindsey & Yeoman (1986). The most popular methods are entrappment in beads of calcium alginate or agarose. Cells entrapped in these gels remain viable and produce appreciable levels of secondary metabolites. However, the culture medium must completely inhibit growth (Brodelius 1983) to prevent the gel beads from breaking up, and calcium alginate requires a high concentration of calcium ions and a low phosphate concentration to preserve its stability.

Immobilization in polyurethane foam which was described for plant cells by Lindsey *et al* (1983) presents certain distinct advantages. The method is extremely simple which decreases the likelihood of cell damage and microbial contamination. Polyurethane foam blocks ($1cm^3$) are added to freshly subcultured cell suspension cultures and during the culture period that follows the cells enter the foam pores, become entrapped and grow to fill the internal spaces. The packed blocks can then be grown in a shaken liquid medium, as they were in this study, or in columns through which medium is circulated from a reservoir. The foam is non-toxic, robust, autoclavable, stable and cheap. The cells are in direct contact, while in gels the aggregates are isolated from each other. There are no special medium requirments to maintain the stability of the polyurethane; and if the cells are maintained in full growth medium, some cells will be lost from the surface, but the foam particles will not break up. This means that the effects of immobilization *per se* and of growth limitation imposed via the medium can be studied separately.

Polyurethane foam has previously been used to immobilize cells of *Daucus carota* and *Capsicum frutescens* (Lindsey *et al* 1983), and it was necessary to develop and characterize the method for cells of *A. belladonna* and *H. muticus* before further experiments could be conducted.

PRODUCT RELEASE

Release of the desired secondary product into the medium is an essential part of the proposed industrial processes using immobilized cell culture systems, as this facilitates continuous production. Also the continuous or intermittent removal of the product could alleviate any feedback inhibition of the pathway.

Since tropane alkaloids are transported from what is apparently their site of synthesis, the roots, to the aerial parts of the plant, and can be detected in the intercellular spaces as well as in the vacuoles (Verzar-Petri 1973), it is not unreasonable to anticipate that cell cultures will spontaneously release alkaloids into the medium. Without release it is essential to permeabilize the cells at intervals, using a treatment which does not cause undue loss of viability or productive capacity.

Permeabilization

There are many methods for permeabilizing cells (reviewed by Felix 1982) most of which were developed with the aim of allowing the entry into the cytoplasm of low molecular weight compounds to enable studies on biosynthesis. In these studies the viability of these cells on a long term basis was not important and therefore these methods were chosen to maximise permeability. Such an approach is not suitable for inducing secondary product release, as the retention of cell viability and synthetic ability are required. Permeabilization methods fall into two broad categories; chemical and physical.

Chemical Methods

The chemical treatments are very diverse, the types of substances included in this category are:

organic solvents e.g ether, dimethylsulphoxide (DMSO)
membrane active antibiotics e.g. nystatin, filipin
polycations e.g.poly-L-lysine, chitosan
detergents e.g.Triton X-100
calcium chelators e.g.EDTA

Felix *et al* (1981) tested a range of methods for their ability to permeabilize cells of *Catharanthus roseus*. DMSO was selected as the best agent and methods using DMSO were developed further (Felix & Mosbach 1982, Brodelius & Nilsson

1983). It was shown that treatment with 5 % DMSO for 30 min was sufficient to cause the release of 85-90 % of the ajmalicine isomers accumulated in the vacuole. Similarly 7.5 % DMSO permeabilized cells of *Anabaena variabilis* and stimulated the 5-aminolevulinate pathway, whereas methods using mechanical treatments and detergents were unsuitable as they led to the loss of the pathway (Avissar 1983). However, different species vary in their response to DMSO (Brodelius & Nilsson 1983), and Parr *et al* (1984) found that a concentration of > 5 % was required before cells of *Cinchona ledgeriana* began to release alkaloids, and cells which were fully permeabilized (after treatment with 20 % DMSO for 7 hours) were permanently damaged. Therefore the use of DMSO is not a universally applicable permeabilization method.

Variability in species response was also observed with the antibiotic filipin, which induces the release of betacyanin from cells of red beet, but had no effect on cells of apple or carrot (Mudd & Kleinschmidt 1970).

Immobilization in chitosan caused the release of oxalate from *Amaranthus tricolor* and proteins from *Asclepias syriaca* (Knorr *et al* 1985, Knorr & Teutonico 1986). The effect of this treatment on cell viability was not examined, but Young *et al* (1982) showed that a concentration of chitosan above 100 $\mu g ml^{-1}$ caused severe damage to cells of *Glycine max* and similar effects were also found with other polycations.

Thus chemical methods are generally not widely applicable and frequently cause loss of cell viability. The reasons for this could be that; i) in attempting to cause release from the vacuole two membranes must be permeabilized, the plasmalemma and the tonoplast. One of them is likely to be more susceptible to the treatment than the other, this has been shown for DMSO (Delmer 1979) and poly-L-lysine (Lerner & Reuveni 1982), and therefore in permeabilizing both membranes, the more susceptible is likely to be severely damaged. ii) Permeabilization of the tonoplast will probably result in some mixing of the cytoplasm and the vacuolar contents. Since the latter includes phenolics and degradative enzymes this would be expected to be detrimental to the cells (Parr *et al* 1987). A further disadvantage of chemical methods is that there may be difficulty in washing the chemical from the cells, particularly in a large bioreactor.

Physical Methods

Physical methods of permeabilization include osmotic shock, temperature shock, ultrasonication (Felix 1982), high ionic strength (Tanaka *et al* 1985), and alteration of the medium pH (Renaudin *et al* 1985). Some of these methods can be expected to suffer from the drawbacks listed above: ultrasonication could cause mixing of the cytoplasm and vacuolar contents; and temperature shocks may be difficult to apply particularly in a bioreactor, as although constant temperature maintenance would be necessary, rapid evenly distributed changes in temperature would be more difficult to control.

Since an extensive comparison and development of permeabilization methods was not within the scope of this project, a small number of physical methods only were investigated, those selected were osmotic shock, high ionic strength, and alteration of the medium pH. All of these treatments had previously been shown not to reduce cell viability, although only pH treatments caused release of alkaloids from the vacuole. Recently electroporation has been used extensively to permeabilize the plasmalemma and the nuclear membrane reversibly and without affecting viability (e.g. From *et al* 1986), and if it is shown to have similar effects on the tonoplast, it may prove useful as a method of inducing product release.

AIMS AND OBJECTIVES

The aims of this project were to examine tropane alkaloid production *in vitro*, and to identify factors which influence it. In brief the factors examined were growth and cell organization. These aims can be broken down to give the following objectives;

i) To characterize and compare tropane alkaloid production in suspended and immobilized cultures.

ii) To determine the effect of growth inhibition, mediated by nutrient limitation, on alkaloid production.

iii) To find out if alkaloid precursors added to the medium could increase yields, and, if possible to identify the limiting branch or stage of the pathway.

iv) To find out if the basal medium and the growth substances added to it could affect the amount of structural differentiation and the alkaloid yield of cell cultures.

v) To determine if the organization of cell cultures into recognizable plant structures (structural differentiation) can increase the alkaloid yield or affect the pattern of alkaloids produced.

vi) To find a suitable method for permeabilizing cell cultures which causes the release of secondary products into the medium without unduly damaging the cells.

In order to pursue these objectives, it was first necessary to develop an analytical system for the study, and to develop and characterize the immobilization procedure for cells of *A. belladonna* and *H. muticus*.

CHAPTER TWO

MATERIALS AND METHODS

Section 2.1 PLANT MATERIAL

The major species used in this investigation were *Atropa belladonna* and *Hyoscyamus muticus*. Seeds of *A. belladonna* were collected from plants grown in the grounds of the Botany Department, Edinburgh. Seeds of *H. muticus* were obtained from Dr. P. J. King, of the Friedrich Miescher-Institut, Basel, Switzerland (a gift which is gratefully acknowledged).

Preliminary studies also involved *Atropa accuminata*, *A. baetica*, and *A. pallidiflora*. Seeds of these species were obtained from Dr. H. Heltman, of the Institute für Pharmazeutische Biologie, der Universitat Bonn, Federal Republic of Germany (a gift which is gratefully acknowledged); but callus initiated from them did not grow well in the culture media used.

Section 2.2 CELL AND TISSUE CULTURE

Section 2.2.1 PREPARATION OF CULTURE MEDIA

i) Schenk and Hildebrandt medium and Standard Synthetic Medium

Schenk and Hildebrandt medium (SH) (Schenk & Hildebrandt 1972) and Standard Synthetic Medium (SSM) (Thomas & Street 1970, 1972) were made up at ten times the normal concentration and stored at -40°C in 50 ml portions. When required, the concentrated stock medium was diluted with distilled water, growth substances were added, and the pH was adjusted to 5.7, before it was made up to the correct concentration in a volumetric flask.

The constituents of these media are listed in Table 2.2.1. Unless otherwise stated in the text, the growth substances added to SH medium were: 2 mgl^{-1} para-chlorophenoxyacetic acid (CPA), 0.5 mgl^{-1} 2,4-dichlorophenoxyacectic acid (2,4-D), and 0.1 mgl^{-1} kinetin (K). Growth substances added to SSM are described in the text.

ii) Murashige and Skoog medium

Murashige and Skoog (MS) medium was prepared from freeze-dried stock medium supplied by Flow Laboratories Ltd., Irvine, Scotland. (cat. no. 26-100-22), the constituents of which are shown in Table 2.2.1. Stock medium

(4.71 gl^{-1}) was dissolved in distilled water together with sucrose (3 % w/v), and growth substances, then the pH was adjusted to 5.7, before it was made up to the appropriate volume in a volumetric flask. In most of the experiments described in Chapter Three, the growth substances added to MS were; 2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K, and this was described as MS-CDK medium.

iii) MS media lacking in phosphate, in nitrogen, and in phoshate and nitrogen

MS media lacking in nitrogen (MSN medium), in phosphate (MSP medium), and in nitrogen and phosphate (MSNP medium) were used in the experiments described in Section 3.6. The constituents of these media are listed in Table 2.2.1. Their constituents were dissolved in distilled water immediately prior to use. The following growth substances were added; 2 mgl^{-1} CPA, 0.5 mgl^{-} 2,4-D, and 0.1 mgl^{-1} K, and the pH was adjusted to 5.7 before the media were made up to the required volume.

In addition to the above ingredients solid media contained 6 gl^{-1} agar (Oxoid No.1).

Table 2.2.1

The nutrient constituents of all tissue culture media used in this investigation, and their concentration in mgl^{-1}. Schenk and Hildebrandt, SH, medium (Schenk & Hildebrandt 1972), Standard Synthetic medium, SSM, (Thomas & Street 1970, 1972), Murashige and Skoog, MS, medium (as supplied by Flow Laboratories Ltd., Irvine, Scotland. cat. no. 26-100-22), MS medium lacking in nitrogen, MSN medium, in phosphate, MSP medium, and in nitrogen and phosphate, MSNP medium.

Constituents	Concentration in Media (mgl^{-1})					
	SH	SSM	MS	MSN	MSP	MSNP
$CaCl_2.2H_2O$	200	-	440	440	440	440
$CaNO_3.4H_2O$	-	208.5	-	-	-	-
$CoCl_2.6H_2O$	0.1	-	0.025	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.2	0.001	0.025	0.025	0.025	0.025
FeNaEDTA	-	2.9	36.7	36.7	36.7	36.7
$FeSO_4.7H_2O$	15	-	-	-	-	-
$(Na_2)EDTA$	20	-	-	-	-	-
H_3BO_3	5.0	1.5	6.2	6.2	6.2	6.2
KCl	-	845	-	-	-	-
KH_2PO_4	-	-	170	170	-	-
KI	1.0	0.75	0.83	0.83	0.83	0.83
KNO_3	2500	80	1900	-	1900	-
$MgSO_4.7H_2O$	400	720	370	370	370	370
$MnSO_4.4H_2O$	10	7.0	22.3	22.3	22.3	22.3
$NaNO_3$	-	1800	-	-	-	-
$NaSO_4$	-	200	-	-	-	-
$NaH_2PO_4.7H_2O$	-	320	-	-	-	-
$NaMoO_4.2H_2O$	0.1	$1x10^{-3}$	0.25	0.25	0.25	0.25
$NH_4H_2PO_4$	300	-	-	-	-	-
NH_4NO_3	-	-	1650	-	1650	-
$(NH_4)_2SO_4$	-	790	-	-	-	-
$ZnSO_4.7H_2O$	1.0	3.0	8.6	8.6	8.6	8.6
*myo*inositol	1000	100	100	100	100	100
nicotinic acid	5.0	0.5	0.5	0.5	0.5	0.5
thiamine HCl	5.0	0.1	0.1	0.1	0.1	0.1
pyridoxine HCl	0.5	0.1	0.5	0.5	0.5	0.5
glycine	-	3.0	2.0	2.0	2.0	2.0
sucrose	30000	30000	-	30000	30000	30000

Section 2.2.2 STERILIZATION AND STERILE TECHNIQUE

Section 2.2.2.1 Sterilization of Equipment and Media

i) Sterilization by Heat

All glassware, instruments, filtration units, distilled water, pretreated foam blocks, and nutrient media were sterilized by autoclaving at 121°C for 20 min at a steam pressure of 15 psi.

ii) Sterilization by Filtration

This method was used to sterilize medium to which tropane alkaloid precursors had been added. The complete, fully diluted medium, which had been pH adjusted was pumped through a Sterivex-™GS 0.22 µm filter unit (cat. no. SVGSB1010) into a sterile flask, using a Millipore fixed speed peristaltic pump (cat. no. XX80 202 30).

Section 2.2.2.2 Sterilization of Seeds

Seeds, wrapped in a muslin bag, were surface sterilized for 30 min in aqueous sodium hypochlorite, containing 2-2.8 % availiable chlorine (a 20 % v/v dilution of a sodium hypochlorite solution supplied by A. J. Beveridge Ltd. Edinburgh) and three drops of Teepol (as a surfactant). After being rinsed in sterile distilled water three times, under aseptic conditions, they were sterilized again in aqueous hydrogen peroxide containing approximately 1.5 % w/v hydrogen peroxide (a 5 % dilution of an AnalaR hydrogen peroxide solution containing approximately 30 % w/v hydrogen peroxide, supplied by BDH) and three drops of Teepol, for 10 min. The seeds were then removed under aseptic conditions and rinsed three times in sterile distilled water before use.

Section 2.2.2.3 Sterile Technique

All cell culture manipulations were carried out at a laminar flow bench over which a continuous stream of sterile air was passed. The work surface was swabbed, and sprayed with absolute ethanol before and after use. Instruments, while not in use, were stored in ethanol, and flamed immediately prior to use.

Section 2.2.3 INITIATION AND MAINTENANCE OF CULTURES

Section 2.2.3.1 Culture Conditions

All cell cultures were grown under the following conditions:

Temperature	25 +/- 2°C
Photon flux density	25 $\mu molm^{-2}sec^{-1}$
Light source	Compton warmwhite fluorescent
Liquid culture agitation	Continuous rotation in a horizontal plane, 98 rpm, 8 mm amplitude

Section 2.2.3.2 Initiation of Callus Cultures

Callus cultures were grown either in 200 ml screwtop jars (stock cultures only) containing 50 ml solid medium, or in 9 cm polystyrene Petri-dishes (Sterilin) containing 20-25 ml solid medium. The Petri-dishes were sealed with parafilm (American Can Company) to exclude microorganisms and to prevent desiccation.

Sterilized seeds were plated onto MS medium containing 5 mgl^{-1} 2,4-D. *H. muticus* seeds germinated and directly formed green, friable callus. *A. belladonna* seeds did not form callus directly, despite the high concentration of 2,4-D. Therefore the germinated seedlings were cut into pieces of 2-3 mm and plated onto fresh medium. After 4-6 weeks some of the explants, usually pieces of root, had formed callus.

Section 2.2.3.3 Initiation of Cell Suspension Cultures

Cell suspension cultures, unless stated otherwise in the text, were grown in 250 ml conical (Erlenmeyer) flasks, containing 50 ml liquid medium, and covered with a double layer of aluminium foil. They were initiated by adding 1-2 g (f. wt.) of friable callus to liquid medium in a conical flask which was then placed on an orbital shaker (Section 2.2.3.1).

Section 2.2.3.4 Maintenance of Callus and Suspension Cultures

All stock cultures were maintained in SH medium. A perforated spoon spatula was used to transfer 1-2 g of callus to fresh medium. Where there were differences in phenotype between cells within a culture, e.g. in friability, greening, or anthocyanin content, care was taken to transfer representatives of all cell types. The interval between subculturing was 4 weeks for *A. belladonna*, and 2-3 weeks for *H. muticus*.

Cell suspension cultures grew to such a high density, that they too were transferred to fresh medium with a perforated spoon spatula. 1-2 g wet weight of cells was transferred at intervals of 3-4 weeks for *A. belladonna* and 2-3 weeks for *H. muticus*.

Section 2.2.4 IMMOBILIZATION OF SUSPENDED CELLS

Cell suspension cultures were immobilized in blocks of foam as described by Lindsey *et al* (1983). Polyether-type polyurethane foam, cut into 1.0 cm^3 blocks, was supplied by Declon Ltd. (Corby, UK). The pore size of the foam, unless otherwise stated in the text, was 40 pores per inch (ppi).

Section 2.2.4.1 Pretreatment of the Foam Blocks

The foam blocks were boiled in distilled water for one hour, and rinsed twice, first in water then in ethanol, to remove any toxic substances. The number of blocks required for each 250 ml culture flask (see text) was then placed in a 50 ml plastic Corning centrifuge tube. The blocks were sterilized in these tubes by heat as described in Section 2.2.2.1.i).

Section 2.2.4.2 Immobilization Procedure

Sterile blocks were added to a freshly subcultured suspension culture. The flask was agitated vigorously in order to submerge the foam, then placed on an orbital shaker. During the following 2-4 weeks cells became entrapped then grew and divided within the foam. At the end of the culture period, when the blocks were densely packed with cells, they were transfered to fresh medium, or analysed for growth and alkaloid content.

Section 2.3 MEASUREMENT OF CULTURE GROWTH AND VIABILITY

Section 2.3.1 MEASUREMENT OF CULTURE GROWTH

Section 2.3.1.1 Measurement of Culture Wet Weight and Fresh Weight

The wet weight of suspended and immobilized cells was determined immediately after their removal from liquid culture media. The fresh weight (f. wt.) of suspended and immobilized cells was measured after excess medium had been removed by filtration through Whatman filter paper No. 1, at reduced pressure, using a Buchner funnel. The fresh weight of immobilized cells was determined by weighing the foam block containing cells, and subtracting the mean weight of a foam block which had been saturated in medium then filtered as above.

Section 2.3.1.2 Measurement of Culture Dry Weight

The dry weight of cells was measured after excess medium had been removed (Section 2.3.1.1), and they had been heated at 60°C for 18 hours, in a hot air oven. The dry weight of immobilized cells was determined by weighing the dried block and cells together, then subtracting the mean weight of a dried foam block.

Section 2.3.1.3 Determination of Cell Number

The cell population density of suspended cells and the number of cells in the foam blocks was estimated with a Hawksley Crystallite Haemocytometer (grid volume 1.8 µl). Cell aggregates were dispersed and foam blocks were broken down as described by Lindsey *et al* (1983) (after the method of MacCarthy *et al* (1980)). Two ml of a thoroughly mixed suspension culture, or one foam block was incubated in 15 % w/v chromium trioxide solution for 30 min at 70°C, then cooled and shaken for 1-2 min. Six grids were counted for each sample, the mean of which was used to estimate the density of the suspended cell population or the immobilized cell number. Results have been expressed in terms of suspended and immobilized cells per culture flask.

Section 2.3.1.4 Calculation of the Relative Growth Index

The growth of plant cell cultures has been expressed as the relative growth index, I_{RG}. It was calculated using the following equation:

$$I_{RG} = (W_1 - W_0) / W_0$$

Where:

W_0 = original weight of cells (g wet wt. or f. wt., mg dry wt.)

W_1 = final weight of cells (g wet wt. or f. wt., mg dry wt.)

Section 2.3.2 DETERMINATION OF CULTURE VIABILITY

In preliminary investigations, the method of Widholm (1972) which employs fluorescein diacetate to stain "viable" cells, was used with *A. belladonna*cultures. It was rejected as there was no clear distinction between fluorescing and non-fluorescing cells. Instead the specific respiration rate, K_R, (Tanaka *et al* 1985) was adopted as an indication of the viability of cell cultures.

Section 2.3.2.1 Determination of the Specific Respiration Rate

An oxygen electrode (Rank Brothers, Cambridge, UK) connected to a chart recorder was used to measure the rate at which cells absorbed oxygen (O_2) from distilled water. Each time the oxygen electrode was used after having been switched off, it was calibrated as described below.

Air was vigorously bubbled through distilled water for at least one hour. The concentration of oxygen at ambient temperature (25°C) was then assumed to be 258 $nmolml^{-1}$. Five ml of oxygen saturated water was added to the chamber of the oxygen electrode, and the level on the chart recorder was set at maximum. The dissolved oxygen was then removed from the water by adding 2-5 mg of sodium dithionite, and the zero position on the chart recorder was set.

The rate of oxygen uptake of cells was determined by adding 0.5 g fresh weight of suspended cells or one foam block, containing cells, cut into 25-30 pieces, to 5 ml of oxygen saturated water in the chamber, and recording the oxygen concentration of the water for 20-30 min. The specific respiration rate, K_R, was calculated using the following equation.

$$K_R = \frac{(C_0 - C_1)\ 258}{(T_1 - T_0)\ C_0.W}$$

Where:

K_R = specific respiration rate ($nmolO_2 ml^{-1} min^{-1} g^{-1}$)

T_0 = time at beginning of measurement (min)

T_1 = time at end of measurement (min)

C_0 = reading on chart recorder at T_0 (cm)

C_1 = reading on chart recorder at T_1 (cm)

W = fresh weight of cells added to the chamber (g)

Section 2.4 CHEMICAL ANALYSIS OF TROPANE ALKALOIDS

Section 2.4.1 EXTRACTION OF TROPANE ALKALOIDS

Section 2.4.1.1 Extraction of Tropane Alkaloids from Plant Cells

This procedure was derived from those described by Phillipson & Handa (1976), Hiraoka *et al* (1973) and Baumann (personal communication). The development of this method is described in Section 3.1.1.

Before maceration, foam blocks were cut into 20-25 pieces. Tissue was macerated in 5 % v/v ammonia (an ammonia solution, AnalaR, containing about 35 % NH_3) in methanol then left to stand overnight. The filtered extract was concentrated, under reduced pressure, at 45°C, and made up to 15 ml with 0.1 M hydrochloric acid. The acid extract was filtered and made alkaline (pH 9) with approximately 2 ml of a buffer, composed of 10 ml 0.2 M ammonium chloride and 9.6 ml of 25 % v/v ammonia. It was then made up to 20 ml with distilled water, and added to the top of an "Extrelut" column (Merck). After 20 min the column was eluted with 40 ml of chloroform. This chloroform extract was evaporated to dryness, under reduced pressure, at 60°C, and the residue taken up in methanol before being stored at 5°C.

Section 2.4.1.2 Extraction of Tropane Alkaloids from Culture Medium

The method used was that of Hiraoka *et al* (1973). A known volume (x ml) of medium was filtered through Whatman filter paper No.1 and made alkaline (pH 9) using the buffer described in Section 2.4.1.1. It was then extracted in a separating funnel three times with x ml of chloroform. Dissolved water was

removed from the chloroform extract with anhydrous sodium sulphate. It was then evaporated to dryness, under reduced pressure, at 60°C, and the residue was taken up in methanol before being stored at 5°C.

Section 2.4.2 THIN LAYER CHROMATOGRAPHY

Thin Layer Chromatography (TLC) was used to perform qualitative, and semi-quantitative analysis of the extracts.

Section 2.4.2.1 Thin Layer Cromatography

Plastic backed TLC plates (20 x 20 cm) coated with Kieselgel-60 (Merck) were used as the adsorbent. The solvent system; ethylacetate: *iso*propanol: 20% v/v aqueous ammonia (45: 35: 15) (Phillipson & Handa 1975) was used routinely to separate the alkaloids. When necessary, the qualitative analysis was confirmed by running the extract again in one of the following solvent systems; acetone: water: ammonia (90: 7: 3) (Phillipson & Handa 1975), or methanol: ammonia (100: 1.5) (Clarke 1970).

Loaded TLC plates were eluted for 1-1.5 hours in a sealed tank which had been equilibrated for 1-2 hours. When dry the plates were sprayed with Dragendorff's reagent, or (more rarely) with Iodoplatinate reagent (Section 2.4.2.2).

Alkaloids in extracts were identified by their co-elution with alkaloid standards, which were run in parallel with the extracts on each TLC plate. A semi-quantitative measure of the amounts of alkaloids present in the extracts was gained by comparing the sizes of the spots with those produced by known amounts of alkaloid standards.

Section 2.4.2.2 Alkaloid Detecting Reagents for TLC

i) Dragendorff's Reagent

Two concentrated stock solutions were prepared as follows;

Solution A. 0.85 g of bismuth oxynitrate were dissolved in 10 ml of glacial acetic acid and 40 ml water was added.

Solution B. 16 g of potassium iodide were dissolved in 40 ml of water.

These solutions could be stored, at 5°C, for a number of months.

Immediately before use, Dragendorff's reagent was prepared by mixing 2.5 ml each of solutions A and B, and adding 25 ml of 20 % v/v aqueous glacial acetic acid. The reagent stains tropane alkaloids orange, tropine pale pink, and the background yellow.

ii) Iodoplatinate Reagent

The reagent was prepared by adding 10 ml of 5 % w/v aqueous platinum chloride, and 5 ml of concentrated hydrochloric acid to 240 ml of 2 % w/v aqueous potassium iodide. It was filtered through Whatman filter paper No.1, and stored at 5°C until required. Atropine and scopolamine were detected immediately after plates were sprayed as purple spots which later faded a little, atropine-N-oxide was only detected after the plate had dryed, as a magenta spot. Tropine gave a pale pink spot on the gray-pink background.

Section 2.4.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) was used to perform both qualitative and quantitative analysis of extracts.

Section 2.4.3.1 Preparation of the Mobile Phase

All solvents used were of HPLC grade (BDH or Fisons). They were filtered at reduced pressure to remove any particles which could block or damage the HPLC pumps. Aqueous solvents were filtered through 0.45 µm pore cellulose acetate membrane filters (Schleicher and Schuell), organic solvents through 0.45 µm pore Nylon-66 membrane filters (Rainin). Immediately before use dissolved

air was expelled from solvents by gently bubbling helium through them from a sparger for 10 min. This was a precaution against air bubbles developing within the HPLC equipment when the solvents were subjected to large pressure differences. While in use, solvent reservoirs were sealed with parafilm to keep out dust particles, and to retard the build up of dissolved air.

Section 2.4.3.2 Preparation of Samples

All extracts were stored in methanol at 5°C. Prior to HPLC the methanol was evaporated to dryness, under reduced pressure at 40°C, and the residue was taken up in the required amount of mobile phase (22.5 % acetonitrile and 77.5% aqueous buffer as described in Section 2.4.3.3). The extract was then filtered, to protect the injection valve, using a Bioanalytical Systems Microfilter fitted with a 0.45 µm pore Nylon-66 membrane filter (Rainin), then placed in a rubber sealed auto-sampler vial (Gilson).

Section 2.4.3.3 HPLC Analysis of Samples

The HPLC system was derived from that of Baumann (personal communication) and was described by Collinge & Yeoman (1986). The development of the HPLC system is described in Section 3.1.2.

HPLC analysis was performed using a Gilson 302 liquid chromatograph, fitted with a Gilson 301 autosampler, a Gilson *u.v.* detector, and a Shimadzu Chromatopac C-RIB data processor. Samples were separated at ambient temperature on a Spherisorb S5 Octyl 25 cm x 4.6 mm column (Phase Separations Ltd. - a gift which is gratefully acknowledged). The column was eluted at 1 $mlmin^{-1}$ with a mobile phase consisting of acetonitrile and a buffer, which contained 50 $mmoll^{-1}$ potassium dihydrogen orthophosphate and was adjusted to pH 3.0 with orthophosphoric acid. Standard samples and whole plant extracts were separated with an isocratic system consisting of 22.5 % acetonitrile and 77.5 % aqueous buffer. Tissue culture and growth medium extracts were more complex, and more concentrated than plant extracts. To decrease the running time for these samples, the gradient system shown in Fig. 2.4.1 was used, as it reduced the retention time of those compounds which were eluted after the alkaloids. Detection was at 254nm. The data processor plotted a chromatogram (absorbance against time), and calculated the area beneath each peak.

Fig. 3.1.10 shows the separation of standards of atropine, scopolamine, and atropine-N-oxide. The area beneath each peak is proportional to the amount of that alkaloid in the sample. Figs. 2.4.2 and 2.4.3 show chromatograms of extracts from leaf material of *A. belladonna* (Fig.2.4.2) and *H. muticus* (Fig. 2.4.3). The identity of alkaloid peaks from tissue extracts was confirmed by the addition of an internal standard to the sample. Fig. 2.4.4 illustrates this procedure. The extract alone was analysed first (Fig.2.4.4.i) and the hyoscyamine peak tentatively identified by its retention time. Then a standard solution of hyoscyamine was mixed with the extract, resulting in the chromatogram shown in Fig. 2.4.4.ii, where the area of the previously marked peak has increased, indicating that it was, indeed, hyoscyamine. The identity of this peak was also confirmed by spectral analysis of the peak using the diode array spectrophotometric detector of a Hewlett Packard 1090 liquid chromatograph.

The area of the atropine peak was proportional to the amount injected, as can be seen in Fig. 2.4.5, which shows the calibration curve for the HPLC determination of atropine. The area of this peak varied from day to day (+/- 200 machine units), and therefore the amount of atropine in samples was not determined using this graph. Instead, the following formulae were used:

$$A_I = P_I F_S$$

Where:

A_I = the amount of atropine (μg) injected in a certain volume, V_I (μl), of the extract

P_I = the area of the resulting peak (machine units)

F_S = the atropine standard response factor, which was determined each day, as follows:

$$F_S = A_S / P_S$$

Where:

A_S = the amount of atropine in a known standard sample (μg)

P_S = the area of the peak produced by A_S μg of atropine (machine units)

The concentration of alkaloid in the extract, C_E $mgml^{-1}$, is then given by:

$$C_E = A_I \times 1000 / V_I$$

And the original concentration of atropine in the tissue, C_T $\mu g g^{-1}$ fresh weight, was:

$$C_T = \frac{A_J \times V_E \times 1000}{V_I \times W_E \times R}$$

Where:

V_E = the total volume of the extract (ml)

W_E = the fresh weight of the tissue extracted (g)

R = 0.73 = the recovery factor, as determined in Section 3.1.1.3

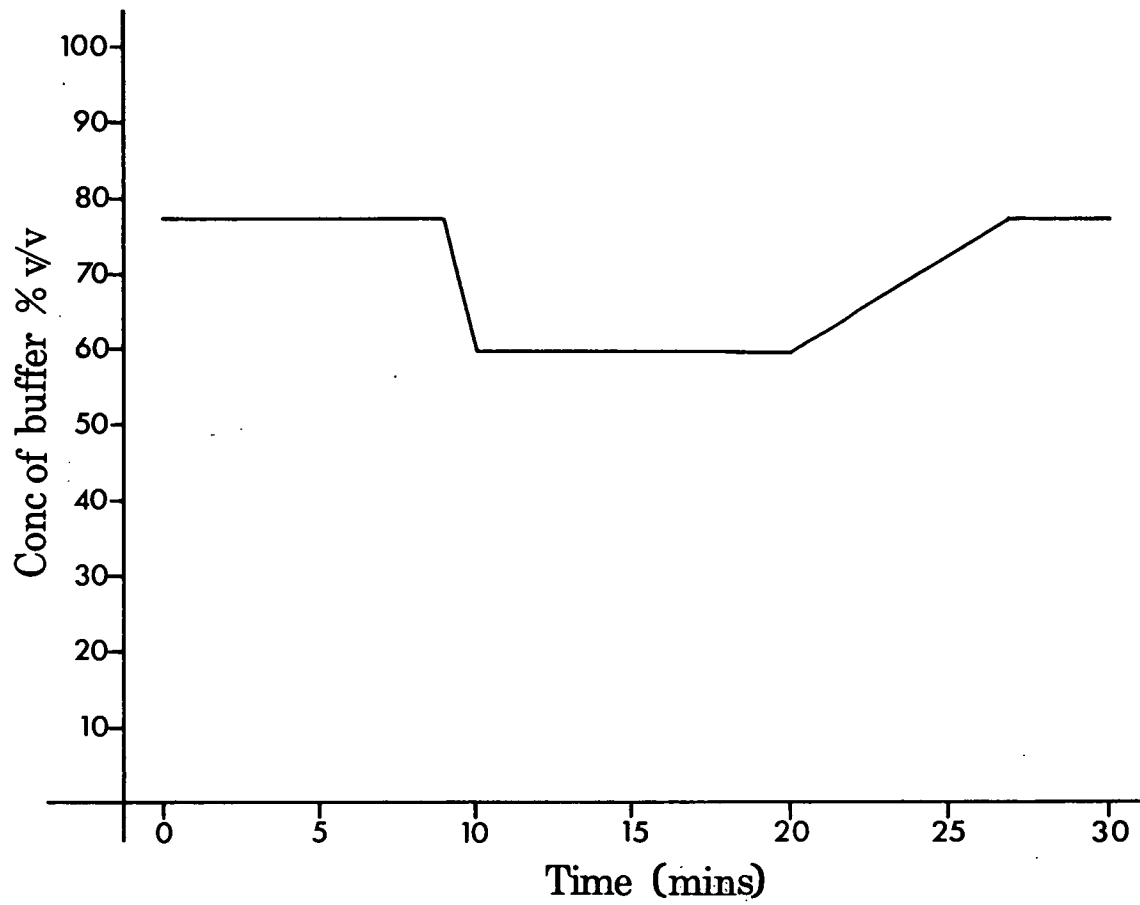
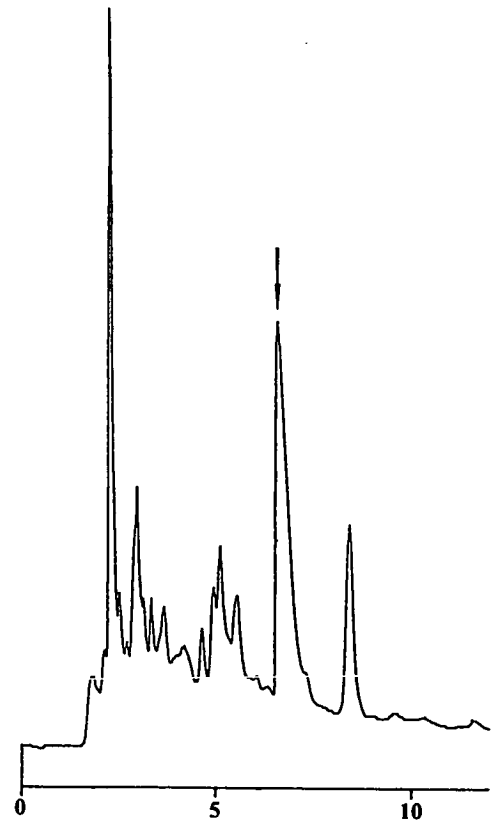


Fig. 2.4.1

The mobile phase gradient system used for the analysis of cell culture extracts by HPLC. The mobile phase consisted of MeCN and aqueous 50 mM KH_2PO_4 buffer at pH 3.0. The graph shows the change, with time, of the proportion of buffer in the mobile phase (% v/v).

Fig. 2.4.2
An HPLC chromatogram of an extract from leaf tissue of *A. belladonna*. The arrow marks the atropine peak. The atropine content of the tissue was 0.38 mgg^{-1} dry. wt..

Fig. 2.4.3
An HPLC chromatogram of an extract from leaf tissue of *H. muticus*. The arrow marks the atropine peak. The atropine content of the tissue was 0.45 mgg^{-1} dry. wt..


0
5
10

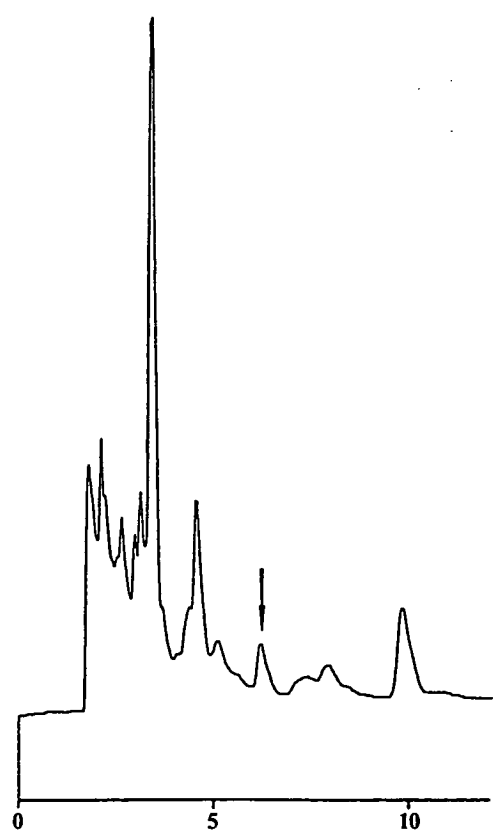
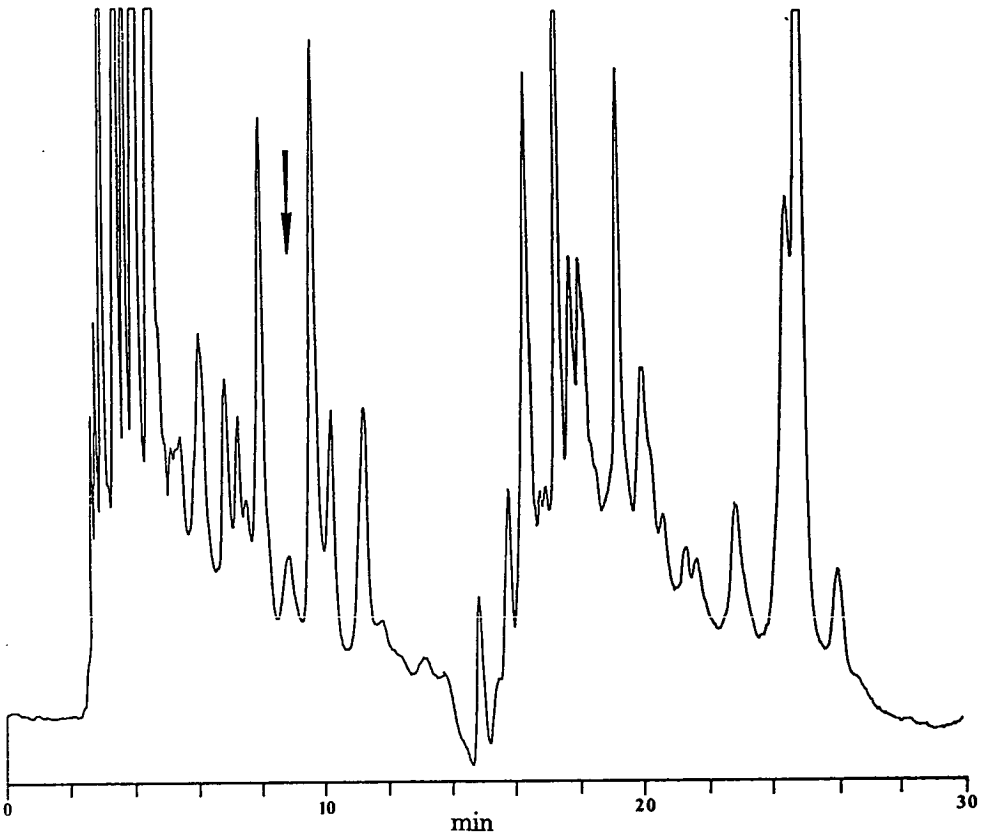

0
5
10

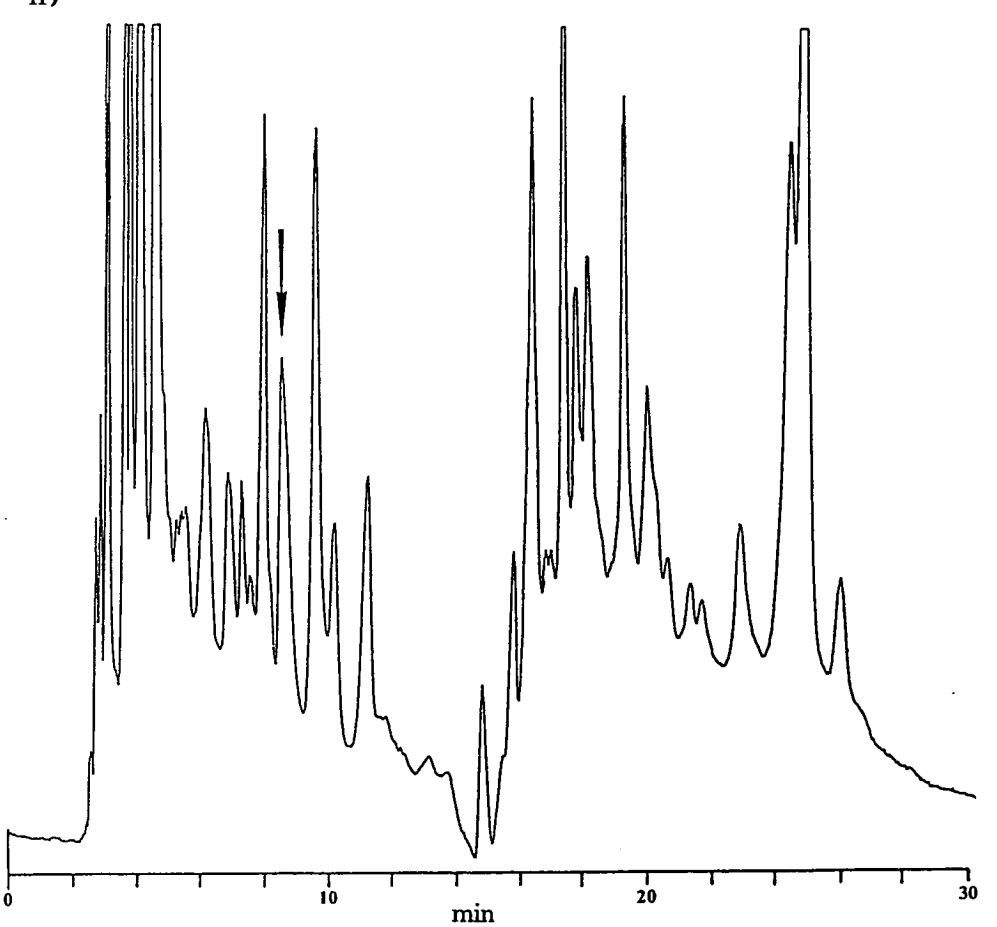
Fig. 2.4.4

The identification of the atropine peak in an extract of immobilized cells of *H. muticus* using the internal standard method. i) shows the HPLC chromatogram of a 10 µl injection of the extract alone; ii) shows the HPLC chromatogram of a 20 µl injection of the same extract mixed in equal proportions with a 0.4 $mgl^{-1}ml$ solution of atropine dissolved in the mobile phase. The atropine peak is marked with an arrow in both cases.

i)



ii)



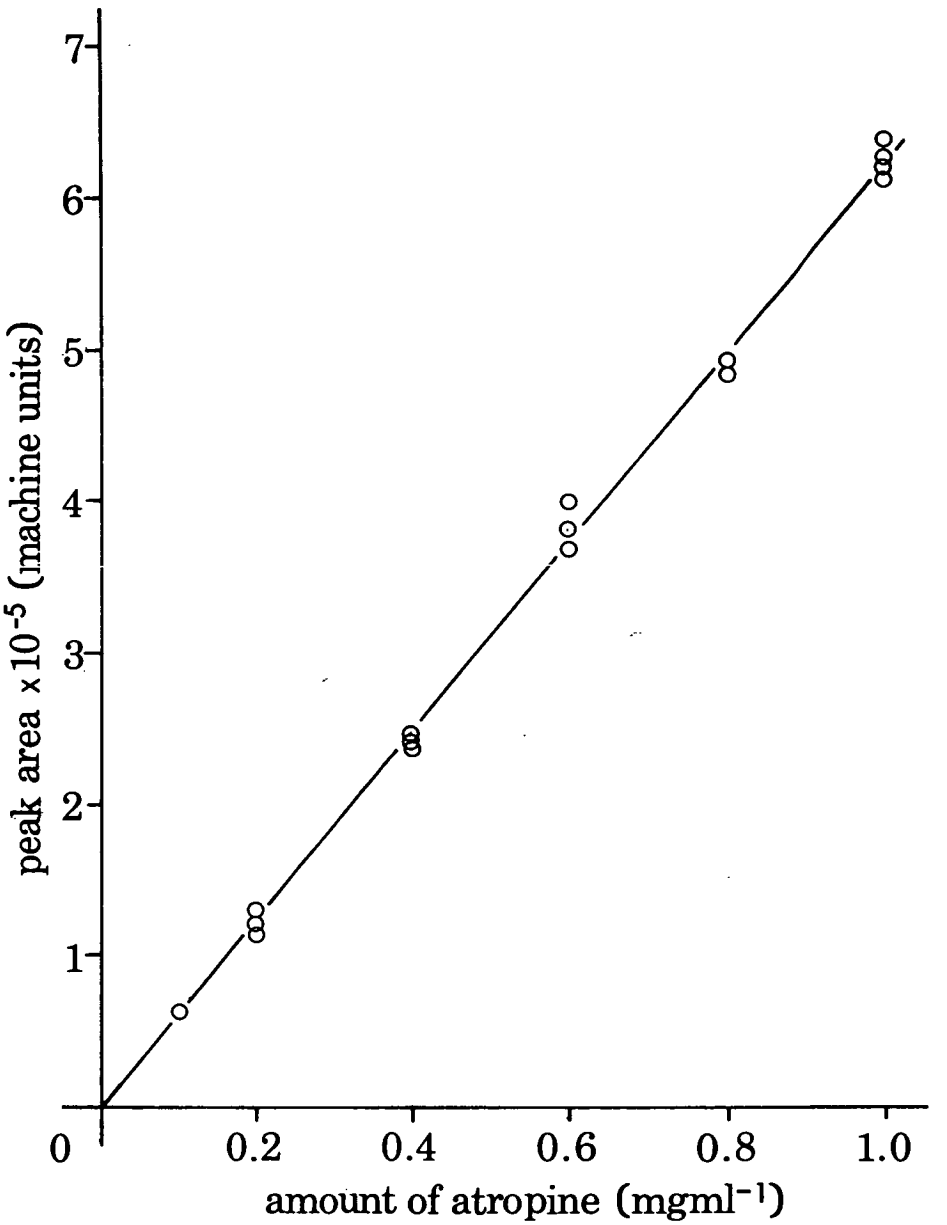


Fig. 2.4.5

The relationship between the amount of atropine in a sample analysed by HPLC (the concentration of atropine in an injection volume of 20 µl) and the area of the resulting peak (machine units), when detected at 254 nm.

Section 2.5 ANALYSIS OF THE ANTHOCYANIN CONTENT OF CELL CULTURES OF *HYOSCYAMUS MUTICUS*

Section 2.5.1 THE EXTRACTION AND DETERMINATION OF ANTHOCYANINS IN CELL CULTURES OF *HYOSCYAMUS MUTICUS*

This method was derived from that of Hall (1984).

Before maceration, excess medium was removed from suspended cells by filtration through Whatman No1 filter paper under reduced pressure, using a Buchner funnel. A known fresh weight (0.25-0.5 g) of fresh tissue was macerated at 4°C with approximately 5 ml of cold (4°C) 1 % v/v concentrated hydrochloric acid in methanol (1 % HCl in MeOH), and left to stand for 4-6 hours at 4°C. The macerate was centrifuged and the pellet was washed twice with approximately 2 ml of cold 1 % HCl in MeOH, the supernatant being conserved. The extract was made up to 10 ml in a volumetric flask with cold 1% HCl in MeOH and mixed thoroughly.

Immediately after dilution, the optical density was measured at λmax, 540nm, with a Pye-Unicam SP 8-100 spectrophotometer, using 1 % HCl in MeOH as the blank. Results have been expressed in OD units/g f. wt./10 ml solvent, each value being the mean of three replicate tissue samples.

Section 2.5.2 THE DETERMINATION OF THE PERCENTAGE OF PIGMENTED CELLS IN CELL SUSPENSION CULTURES OF *HYOSCYAMUS MUTICUS*

The red-purple *H. muticus* suspension cultures when observed microscopically were seen to be composed of both pigmented and non-pigmented cells. Before the proportion of pigmented cells could be determined it was necessary to break up the cell aggregates, so the cultures were subjected to a protoplast isolation procedure.

Suspension culture cells (approximately 0.5 g wet weight) were incubated at 25°C in 5 ml of enzyme solution in a 10 ml vial on a rotary shaker. The enzyme solution contained 4 % cellulase ("Onozuka" R-10) and 2 % macerozyme (R-10, Yakult Pharmaceutical Industry Co., Ltd. Japan) dissolved in a solution of 0.25 M mannitol and 0.1 M calcium chloride at pH 5.7. This solution

before use under reduced pressure through a Whatman glass fibre filter.

A drop of the resulting protoplast suspension was placed between a glass slide and cover slip for observation under the microscope. The percentage of red cells in a sample of 500-600 cells seen in randomly selected fields of vision was determined.

Section 2.6 STATISTICAL ANALYSIS

All methods of statistical analysis, and the required distribution tables were taken from Bailey (1981).

Section 2.6.1 COMPARISON OF THE MEANS OF TWO SAMPLES

Observed differences between means were assessed using "Student's t-test", as it is suitable for the small samples ($n = < 30$) used in this investigation.

The first sample contained n_1 observations and had a mean $\bar{x}_1$, and a variance s_1^2. In the second sample the corresponding values were n_2, $\bar{x}_2$, and s_2^2. The Null Hypothesis was tested that the true means μ_1 and μ_2 were equal. First the common sample variance s_c^2 was calculated:

$$s_c^2 = \frac{[(n - 1)s_1^2 + (n - 1)s_2^2]}{(n_1 + n_2 - 2)}$$

This value was then used to calculate t as follows:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{s_c^2(1/n_1 + 1/n_2)}}$$

The table of "Student's" t-distribution was then referred to at $(n_1 + n_2 - 2)$ degrees of freedom. If t was bigger than the tabulated corresponding to P, the hypothesis of equal means was rejected at the 100P % level of significance, otherwise it was accepted at 100P %.

Section 2.6.2 REGRESSION ANALYSIS

Section 2.6.2.1 Fitting the Regression Line

Regression analysis was performed to determine if there was an association between two measurements, x and y. The assumption was made that the true means of y for each value of x lay on a straight line. This line shows the linear regression of y on x, and is termed the regression line. Its equation is:

$$y = \alpha + \beta x$$

Where β is the regression coefficient of y on x. It may be positive or negative, or zero if y and x are not related.

In order to fit a regression line to the data, the values of the following expressions were calculated.

n			a)
Σx	Σy		b)
Σx^2	Σy^2	Σxy	c)
$1/n(\Sigma x)^2$	$1/n(\Sigma y)^2$	$1/n(\Sigma x)(\Sigma y)$	d)
$\Sigma(x - \bar{x})^2$	$\Sigma(y - \bar{y})^2$	$\Sigma(x - \bar{x})(y - \bar{y})$	e)
s_x^2	s_y^2	c	f)

The values in row e) were calculated by subtracting the values of each expression in row d) from the one above it. Dividing the values in row e) by (n - 1) gave the values in row f), which were then used to calculate the regression coefficient. β is the true regression coefficient and was estimated by b:

$$b = \frac{c}{s_x^2}$$

and the constant α was then estimated by:

$$a = \bar{y} - b\bar{x}$$

The fitted regression line is thus:

$$y = a + bx$$

Section 2.6.2.2 Testing the Significance of the Regression Coefficient

In order to find whether the association between two measurements indicated by the fitted regression line really existed, a test was made to determine if the regression coefficient was significantly different from the hypothetical value zero. Since n = < 30 a t-test was used.

First the variance about the regression line was calculated:

$$s^2 = 1/(n - 2)\left\{\Sigma(y - \bar{y})^2 - \frac{[\Sigma(x - \bar{x})(y - \bar{y})]^2}{\Sigma(x - \bar{x})^2}\right\}$$

This was entered into the following equation to find the value of t:

$$t = \frac{b}{\sqrt{s^2 \,/\, \Sigma(x - \bar{x})^2}}$$

The table of "Student's" t-distribution was then entered at n - 2 degrees of freedom. If t was greater than the tabulated value in the column corresponding to P, the regression coefficient was considered to be different from zero at 100P %, and if t was less than the tabulated value the regression coefficient was not different from zero, and y and x were not related, at the 100P % level of significance.

CHAPTER THREE

RESULTS

The aims of this project were to examine the production of tropane alkaloids *in vitro*, and to develop ways of increasing the atropine yield which would be compatible with a system for its production on an industrial scale.

Before these objectives could be attained, an appropriate experimental system had to be established, and this is described in Sections 3.1 to 3.3. First analytical methods were developed to determine the tropane alkaloid content of cell cultures (Section 3.1). Then procedures were established with immobilized cell cultures using polyurethane foam to enable the study of alkaloid accumulation. Section 3.2 describes the application and optimization of this immobilization method to *Hyoscyamus muticus* and *Atropa belladonna*. The ability of the two species, *A. belladonna* and *H. muticus* which are commonly used as a commercial source of atropine for pharmaceutical purposes, to produce alkaloids in immobilized culture was compared, and the highest producing species selected as the subject of further experiments (Section 3.3).

A series of experiments are presented in the following sections in which attempts were made to control the production of alkaloids, beginning with an experiment to observe their production with time, together with a comparison of the levels accumulated in immobilized and suspended cell cultures (Section 3.4). Then the effects on yield caused by the following changes in the culture medium were examined: i) the addition of alkaloid precursors to the medium (Section 3.5); ii) the limitation of certain nutrients (Section 3.6); iii) the manipulation of added growth substances in a number of basal media (Section 3.7). The experiments in Section 3.7 were designed to examine the effect of morphogenesis on alkaloid production, and this was pursued further in Section 3.8, using lines selected for their increased ability to differentiate.

Finally, because a commercial system for the production of secondary metabolites using immobilized cells requires the release of the product into the medium, attempts were made to permeabilize cells of *H. muticus*, and these are described in Section 3.9.

Section 3.1 DEVELOPMENT OF ANALYTICAL METHODS FOR THE MEASUREMENT OF TROPANE ALKALOIDS IN CELL CULTURES

The development of analytical methods for the detection and measurement of tropane alkaloids in cell cultures involved the selection of an extraction method from existing procedures (Section 3.1.1), and the development of a method to determine the amounts of tropane alkaloids in these extracts (Section 3.1.2). The procedures derived from these experiments are described in Sections 2.4.1.1 and 2.4.3.3 respectively.

The selection of the extraction procedure is described first, although, the two investigations were carried out concurrently. High Performance Liquid Chromatography (HPLC) was used to analyse the extracts for the reasons given in Section 3.1.2.3. Published HPLC methods were tried first and then modified to obtain a clear separation and accurate determination of the major tropane alkaloids in extracts from plant cell cultures.

Section 3.1.1 THE SELECTION OF A PROCEDURE FOR THE EXTRACTION OF TROPANE ALKALOIDS FROM CULTURED CELLS

A number of procedures have been described in the literature for the extraction of tropane alkaloids from plants, and/or from plant cell cultures (Hultin & Tursell 1965, Tabata *et al* 1972, Harborne 1973, Phillipson & Handa 1975, Baumann (personal communication)). These procedures were tested first for their ability to extract a known sample of atropine (Section 3.1.1.1). Some of these methods were then rejected, and those retained were assessed, along with new, modified methods, for their ability to extract alkaloids from leaf tissue of *A. belladonna* (Section 3.1.1.2). The best method was then selected, and the recovery of a known amount of added alkaloid from cultured cells was determined as described in Section 3.1.1.3). Finally, an experiment was performed to find out if the polyurethane foam in which cells were immobilized adsorbed the alkaloids (Section 3.1.1.4).

Section 3.1.1.1 A Preliminary Trial of Extraction Procedures for Tropane Alkaloids

Four extraction procedures derived from methods which had been used previously for the extraction of tropane alkaloids from plant tissue, and/or from plant cell cultures were tested in this experiment. Five mg of atropine (Sigma) was subjected to each of the procedures (i to iv), and the resulting extracts were analysed by HPLC.

i) This method was taken from Phillipson & Handa (1975), and Hultin & Tursell (1965). The sample was mixed with 20 ml of 5 % v/v ammonia (a concentrated AnalaR solution containing about 35 % NH_3) in methanol (5 % NH_3 in MeOH) and left to stand overnight in the dark, at room temperature (c. 25°C). The mixture was filtered under reduced pressure through Whatman No1 filter paper, concentrated under reduced pressure at 50°C and taken up in 20 ml of 2 % v/v aqueous sulphuric acid. This acid extract was filtered under reduced pressure through a Whatman glass fibre filter and made alkaline (pH 9) with 5 % v/v aqueous ammonia before being extracted in a separating funnel three times with 20 ml of chloroform. Dissolved water was removed from the chloroform extract by mixing with anhydrous sodium sulphate then filtering the slurry through a Whatman glass fibre filter. The chloroform was evaporated to dryness under reduced pressure at 60°C, and the residue taken up in 5 ml of methanol before storage at 5°C.

ii) This procedure was described by Baumann (personal communication). The sample was mixed with 17 ml of 0.1 M HCl and left to stand overnight in the dark at c. 25°C, then filtered under reduced pressure through Whatman No1 filter paper. The filtrate was made alkaline (pH 9) with a buffer; composed of 10 ml of 0.2 M ammonium chloride and 9.6 ml of 25 % v/v aqueous ammonia; before being made up to 20 ml with distilled water and added to the top of an "Extrelut" column (Merck). The column was allowed to soak for 15 min then eluted with 40 ml of chloroform. The chloroform was evaporated to dryness under reduced pressure at 60°C and the extract taken up in 5 ml of methanol before storage at 5°C.

iii) This procedure was described by Evans & Partridge (1952) and Basey & Wooley (1973). The sample was moistened with 1 ml of distilled water and left overnight in the dark in an airtight container, at c.25°C. The mixture was made alkaline by adding 0.5 g of calcium chloride before being extracted twice with 25 ml of ether. The layers were separated by centrifugation (5 min at 1250 xg). The ether extract was dried with anhydrous sodium sulphate as in method i), the ether removed under reduced pressure at 40°C, and the residue taken up in 5 ml of methanol before storage at 5°C.

iv) This is Harbourne's (1973) general alkaloid extraction method. The sample was mixed with 20 ml of 10 % v/v glacial acetic acid in absolute ethanol, and left to stand for four hours, at c. 25°C. After concentration under reduced pressure at 40°C to approximately 5 ml, the alkaloid was to have been precipitated by dropwise addition of concentrated ammonia, then collected by centrifugation. However no precipitate was seen even after the addition of a large amount of ammonia (20 ml), so no extract was derived from this method.

The extracts derived from methods i), ii), and iii) were prepared and analysed by HPLC as described in Section 2.4.3, the samples being resuspended in 5 ml of the mobile phase, and compared with a standard atropine sample, which was prepared by taking 1 ml of a 1 $mgml^{-1}$ solution of atropine in MeOH and resuspending it in 1 ml of mobile phase, as described in Section 2.4.3.2. The concentration of atropine in the samples, which at 100 % recovery would have been the same as that of the standard sample (taken as $1mgml^{-1}$) is shown in Table 3.1.1.

The use of method iii) resulted in the loss of much of the standard sample. Perhaps, with practice, the extraction of atropine using this method could have been improved, but since methods i) and ii) both gave good recovery, this was not considered necessary, and methods iii) and iv) were rejected.

Method i) gave an acceptable recovery of the sample and was therefore used in the next stage of the investigation together with method ii), which gave apparently complete recovery. The same experiment was also carried out using standard amounts of scopolamine and hyoscyamine-N-oxide, and similar results were obtained.

Four procedures; i), ii), and a modification of each of these methods, were used in the next stage of the investigation, where alkaloids were extracted from leaf tissue of *A. belladonna*. A comparison was made of their ability to recover alkaloid and to eliminate unwanted substances from the extract.

Extraction procedure	Atropine Concentration in Extract $mgml^{-1}$
control	1.0
i)	0.75
ii)	1.05
iii)	0.211

Table 3.1.1
The concentration of atropine in extracts of a standard sample of atropine produced by the extraction procedures described in Section 3.1.1.1.

Section 3.1.1.2 A Comparison of the Ability of a Number of Procedures to Extract Tropane Alkaloids from Leaf Material of *A. belladonna*.

The aim here was to select the best extraction procedure for tropane alkaloids in plant material. Importance was placed not just on good alkaloid recovery, but also on the presence of contaminating substances in the extracts, which can affect the accuracy of both the identification and the determination of the alkaloids; and on the simplicity and rapidity of the procedure.

Leaves of uniform size (5-6 cm long) were harvested from 2 month old plants grown in a greenhouse (16 hour day, 16°C night temperature) from the seeds described in Section 2.1. Eight samples of known f. wt. (5-6 g) were taken, and four were dried at 60°C in a hot air oven for 18 hours. Two samples, one fresh and one dried, were extracted by each of the following four methods.

i) This was the same as method i) described in the last section, except that the tissue was macerated in 20 ml of 5 % NH_3 in MeOH, and the final extract was dissolved in 10 ml of MeOH.

ii) This method was similar to i) but with an additional step - extraction of the aqueous acidic filtrate (Hultin & Tursell 1965; Tabata *et al* 1972). The tissue was macerated in 20 ml of 5 % NH_3 in MeOH and left to stand overnight in the dark at c. 25°C. The mixture was filtered under reduced pressure through Whatman No1 filter paper, concentrated under reduced pressure at 50°C and taken up in 20 ml of 2 % v/v aqueous sulphuric acid. This solution was filtered through a Whatman glass fibre filter under reduced pressure then extracted in a separating funnel with chloroform (3 x 20 ml). The aqueous layer was retained and made alkaline with 5 % aqueous ammonia before further extraction with chloroform (3 x 20 ml). The chloroform extract was retained and dried with anhydrous sodium sulphate. The chloroform was evaporated to dryness under reduced pressure at 60°C, and the residue taken up in 10 ml of MeOH before storage at 5°C.

iii) This is a slightly modified version of method ii) of Section 3.1.1.1. No more than 20 ml of alkaline filtrate could be added to the "Extrelut" column, but when extracting fresh cell cultures, which have a high water content, it was difficult to keep the volume of the extract at 20 ml or less, without substantially reducing the volume of 0.1 M HCl

used to extract the alkaloids. Therefore the tissue was first macerated in 20 ml of 5 % NH_3 in MeOH and left to stand overnight in the dark at c. 25°C. The resulting extract was filtered through Whatman No1 filter paper and concentrated under reduced pressure at 50°C before being taken up in 15-17 ml of 0.1 M HCl. This acid extract was filtered under reduced pressure through a Whatman glass fibre filter and made alkaline (pH 9) using the buffer described previously (Section 3.1.1.1 method ii)), diluted to a volume of 20 ml with distilled water, and added to the top of an "Extrelut" column (Merck). After 15 min, the column was eluted with 40 ml of chloroform. The chloroform was evaporated to dryness under reduced pressure at 60°C, and the residue taken up in 10 ml of MeOH before storage at 5°C.

iv) This method is similar to method iii) and was included to assess the effect of the "Extrelut" column on the composition of the resulting extract. The alkaline filtrate was prepared as in method iii), but instead of being added to a column, it was extracted in a separating funnel with chloroform (3 x 20 ml). The chloroform was dried with anhydrous sodium sulphate, then evaporated to dryness and the residue taken up in 10 ml of MeOH before storage at 5°C.

The extracts were resuspended in 5 ml of the mobile phase and analysed by HPLC as described in Section 2.4.3. The chromatograms are shown in Fig 3.1.1. Only atropine was detectable in the extracts and the amount extracted per gram dry weight of tissue by each method is shown in Table 3.1.2. The latter results are expressed in mgg^{-1} dry weight, the dry weight of the fresh tissue samples being estimated using the mean ratio of fresh to dry weight of the four dried samples.

The assumption was made that the concentration of atropine in the original tissue samples was the same, and that any difference between the values of mg atropine extracted per g dry weight of tissue was due to the extraction procedures. The true concentration of atropine in the tissue was probably about 1 mgg^{-1} dry weight. This was established from the results presented in Section 3.1.1.1, where method i) gave 75 % recovery of the standard sample. The much higher values for the concentration of atropine in the tissue derived from the extracts made by methods ii) and iv) from fresh tissue are probably due to the many impurities present in the samples which affect the accuracy of the HPLC determination (Fig 3.1.1).

Overall the results show that method iii) not only gave better alkaloid recovery than method i) but also produced better extracts i.e. with fewer impurities. Method ii) was an improvement on method i), giving greater recovery of alkaloids while removing contaminating substances, but was still not as good as method iii), as it extracted compounds with similar retention times to atropine which can interfere with the accuracy of its determination. Method iv) produced extracts containing many impurities, thus illustrating the effect of the "Extrelut" column. Method iii) was selected as the best method as it gave relatively pure extracts from plant tissue, good alkaloid recovery, and it was a simple, relatively rapid procedure.

In the next stage of this investigation, a known amount of atropine was added to cultured cells which were then extracted by method iii) to determine the recovery of atropine from cultured cells.

Extraction procedure	mg atropine extracted per g dry weight of tissue	
	fresh tissue	dry tissue
i)	0.708	0.814
ii)	2.090	1.090
iii)	1.091	0.820
iv)	2.827	0.821

Table 3.1.2
The amount of atropine (mgg^{-1} dry weight) extracted from leaf tissue of *A. belladonna* by the extraction procedures described in Section 3.1.1.2.

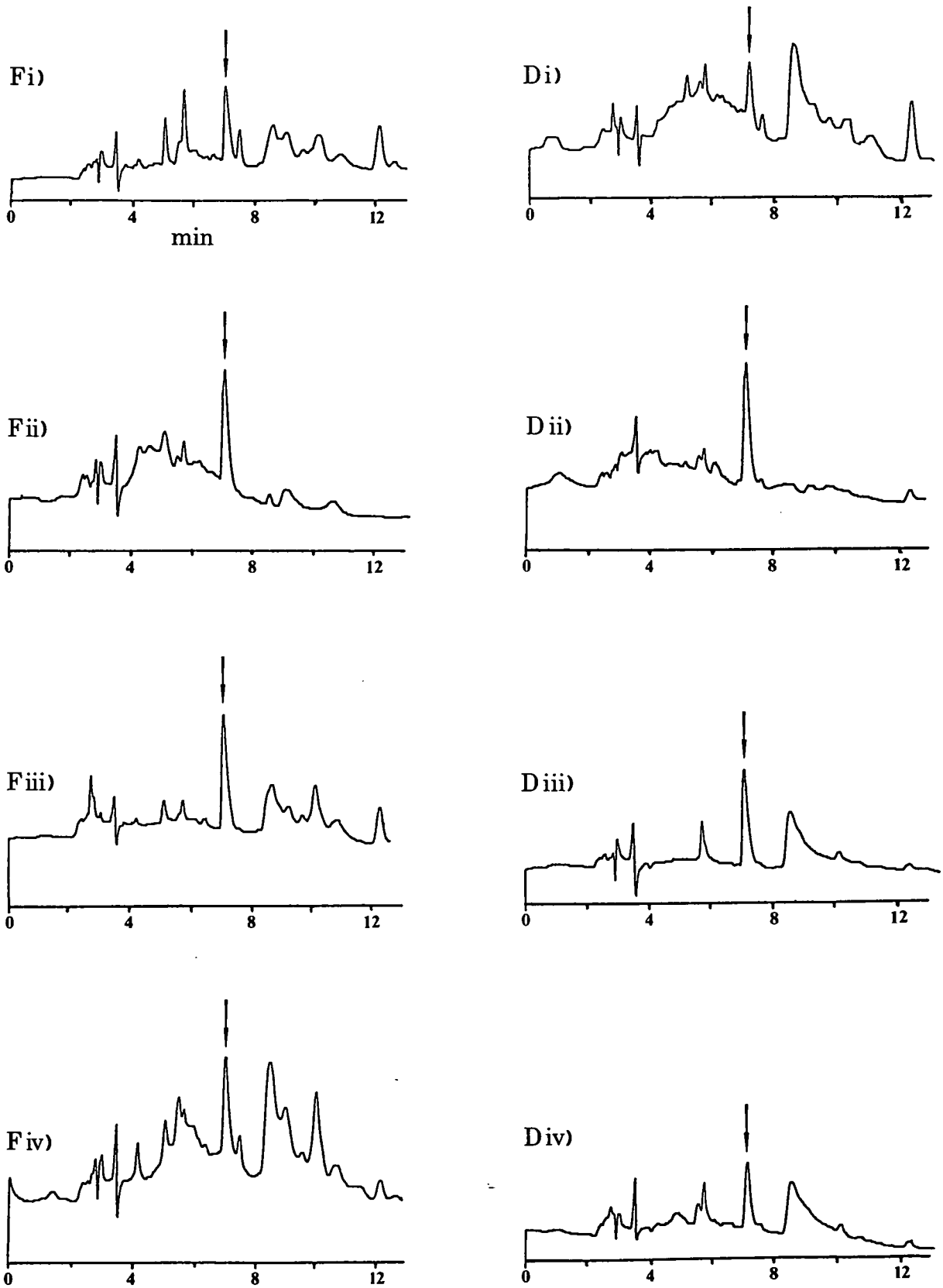


Fig 3.1.1

HPLC chromatograms of extracts from fresh (F) and dry (D) leaf tissue of *A. belladonna* produced by methods i) to iv), described in Section 3.1.1.2. The atropine peak is marked with an arrow.

Section 3.1.1.3 Determination of the Percentage Recovery of Exogenous Atropine from Cell Cultures by the Selected Extraction Procedure

The extraction procedure selected after the last experiment was found in the preliminary test (Section 3.1.1.1) to give apparently complete recovery of alkaloid standards. Here its ability to extract alkaloid standard which had been mixed with cell culture material was examined.

Twenty six day old *H. muticus* callus (from one Petri-dish), grown on SH medium, was divided into two samples, A and B, of known fresh weight (about 10g). Two ml of a stock solution of atropine (1 $mgml^{-1}$ in distilled water) was mixed with B immediately before both samples were extracted as described in Section 2.4.1.1. The extracts were resuspended in 2 ml of mobile phase before being analysed by HPLC as described in Section 2.4.3.

Making the assumption that the samples had the same original alkaloid content, the amount of extracted atropine per g fresh weight of sample A (3.72 μgg^{-1}) was used to calculate the amount of endogenous alkaloid which would have been extracted from sample B. This value was subtracted from the total amount extracted, to show that 1.452 mg of exogenous atropine was extracted from the tissue, i.e. there was 73 % recovery of the added standard.

This incomplete recovery was taken into account when calculating the amount of atropine present in tissue cultures in following experiments; a recovery factor, R = 0.73, being incorporated into the final equation of Section 2.4.3.3.

Section 3.1.1.4 The Extraction of Atropine from the Medium and from Polyurethane Foam

The principle aims of this experiment were to find out if the polyurethane foam adsorbed tropane alkaloids, and if the procedure described in Section 2.4.1.1 could extract bound alkaloid from the foam. It was also used to determine the recovery of alkaloid from the medium by the extraction procedure described in Section 2.4.1.2. Known amounts of atropine were added to SH medium, and to medium containing foam blocks, which were then extracted and analysed by HPLC to determine the amount of atropine recovered.

A standard sample of atropine (1 ml of a 1 $mgml^{-1}$ solution in methanol) was added to 50 ml of SH medium (Section 2.2.1.1), and to 50 ml of SH medium containing five pretreated polyurethane foam blocks (Section 2.2.4.1). Two controls were used, 50 ml of SH without added atropine, and 50 ml of distilled water containing the atropine standard as described above. The latter control was included to determine whether the alkaloid recovery was affected by the constituents of the medium. The four test solutions were placed in 250 ml conical flasks, sealed with aluminium foil and placed on an orbital shaker (Section 2.2.3.1) for 24 hours. The water and medium were then extracted by the method described in Section 2.4.1.2, and the foam blocks by that in Section 2.4.1.1. The samples were prepared as described in Section 2.4.3.2, 1 ml of mobile phase being used to dissolve the extracts, which were then analysed by HPLC (Section 2.4.3.3). The concentration of atropine in the samples is shown in Table 3.1.3. A concentration of 1 $mgml^{-1}$ would indicate complete recovery of the sample.

The recovery of atropine from the medium appears to have been complete, and the SH control showed that there were no substances coeluting with atropine which could have increased its apparent concentration in the extract. The lower amount of atropine in the extract from water suggests that the mean recovery from medium may be less than 100 %.

Approximately 10 % of the atropine added to the medium containing foam blocks was lost and only about 1 % of this was recovered in the foam extract. The rest was probably not bound to the foam, but lost during filtration of the blocks to remove excess medium. Since only a small amount of the atropine added to the medium was subsequently extracted from the foam blocks, the alkaloid content of immobilized cells, determined after extraction by the method in Section 2.4.1.1, would not be deceptively increased by alkaloid which was absorbed from the medium and then extracted from the foam in which the cells were immobilized. However in future experiments, it was assumed that 10 % of the alkaloid in the medium was lost during extraction.

Sample	Conc. of Atropine in Extract $mgml^{-1}$
SH control	0
Water	0.953
SH + atropine	1.010
SH + atropine + foam	0.874
Foam	0.010

Table 3.1.3
The concentration of atropine in the extracts from medium, water, and foam blocks described in Section 3.1.1.4.

Section 3.1.2 THE DEVELOPMENT OF AN HPLC METHOD FOR THE DETERMINATION OF TROPANE ALKALOIDS IN PLANT CELL CULTURE EXTRACTS

A method was required which could separate and determine the major tropane alkaloids of *A. belladonna* and *H. muticus*, i.e. atropine (dl-hyoscyamine), scopolamine, and hyoscyamine-N-oxide, in extracts from plant cell cultures. HPLC has been used to determine these alkaloids, but this was usually in extracts from pharmaceutical products, where the amounts of one alkaloid and its breakdown products were measured. The method of Baumann (personal communication) was developed for the determination of alkaloids in extracts from plant tissue, but not specifically for extracts from plant cell cultures.

The HPLC method described in Section 2.4.3.3 was developed by first performing preliminary tests with methods derived from those described in the literature (Section 3.1.2.1). One of these methods was then selected and changes were made to both the mobile and the stationary phases in an attempt to fulfil the requirements of clear separation and accurate determination (Section 3.1.2.2). The reasons for the choice of HPLC as the method of analysis for this study are given in Section 3.1.2.3.

Section 3.1.2.1 A Preliminary Trial of Previously Published HPLC Methods for the Determination of Tropane Alkaloids

Two previously described HPLC methods were tested in order to establish a starting point, from which a method could be developed for the separation of the major tropane alkaloids in extracts from plant cell cultures.

The method described by Brown & Sleeman (1978) was developed to quantify atropine sulphate and one of its breakdown products, tropic acid, in pharmaceutical preparations. They used an octadecyl-silanol, reversed phase (C_{18}) column, which they eluted at 1.5 $mlmin^{-1}$, with a mobile phase consisting of 35 % acetonitrile, and 65 % aqueous 0.01 M 1-heptanesulphonic acid (an ion-pairing agent), at pH 3.4.

The second method, described by Baumann (personal communication) was developed for the determination of tropane alkaloids in extracts from whole plant material. Their samples were separated at 40°C on a C_{18} column at $1mlmin^{-1}$

with a mobile phase consisting of 17.5 % acetonitrile, and 82.5 % aqueous 50 mM potassium dihydrogen orthophosphate, KH_2PO_4, at pH 2.56.

In this trial investigation, standard samples of atropine, scopolamine, and hyoscyamine-N-oxide were injected onto a 25 cm x 4.6 mm (internal diameter) Ultrasphere Ion-Pair (C_{18}) 10 µm (particle size) column (Anachem), which was eluted at c. 25°C, at 1 $mlmin^{-1}$, with each of the following mobile phases.

(a) 17.5 % acetonitrile, and 82.5 % aqueous 50 mM KH_2PO_4 adjusted to pH 3.0 with orthophosphoric acid.

(b) 35 % acetonitrile, and 65 % 0.01 M 1-heptane-sulphonic acid adjusted to pH 3.4 with HPLC grade glacial acetic acid.

The HPLC apparatus, and the procedures used for the preparation of the mobile phase and the samples are described in (Section 2.4.3). 254 nm was chosen as the detection wavelength from the absorbtion spectrum of atropine shown in Fig 3.1.2.

When the first mobile phase (a) was used, the alkaloid standards were retained on the column, and were not eluted even if the acetonitrile concentration was raised to 80 %. With the second mobile phase (b) the alkaloids were detected only after the acetonitrile concentration was increased to 50 %. Scopolamine was eluted first, then atropine, followed by hyoscyamine-N-oxide. The retention times were long, and the peak shape was very poor, with considerable peak tailing. However, the alkaloids were separated, so this mobile phase was selected for modification as described in the next section.

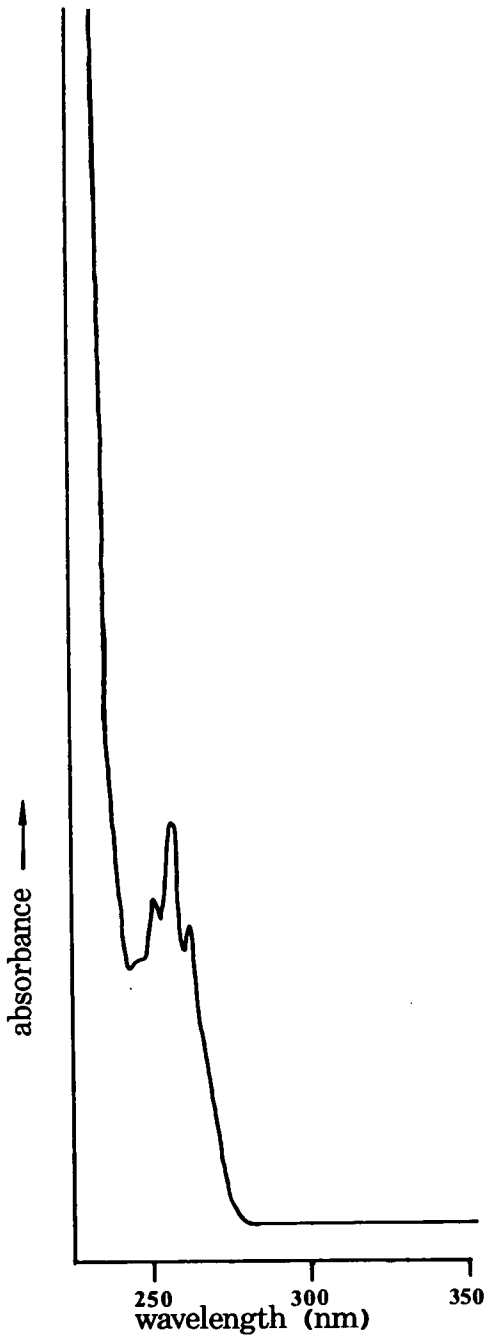


Fig 3.1.2

The absorption spectrum of atropine, scopolamine and hyoscyamine-N-oxide when dissolved in the HPLC mobile phase.

Section 3.1.2.2 The Modification of the Chosen HPLC Method for the Determination of Tropane Alkaloids in Plant Cell Culture Extracts

Once the starting point for the method had been selected, modifications were made in an attempt to satisfy the following criteria; clear separation (resolution) of the alkaloids, good (symmetrical) peak shape, and a short analysis time.

The mobile phase (b) which was selected after the preliminary investigation separated the alkaloids, but peak shape was poor, and the retention times were long. This mobile phase was not buffered which could have led to the proportions of the neutral and the ionized forms of each solute changing throughout its chromatographic band. These two forms of solute would have had different degrees of retention, which could have caused the peak tailing (Knox 1978). Therefore a citric acid:di-sodium hydrogen orthophosphate buffer (Data for Biochemical Research 1969) was incorporated into the aqueous component of the mobile phase, which then contained 15.022 gl^{-1} citric acid, 8.094 gl^{-1} Na_2HPO_4, and 2.022 gl^{-1} 1-heptanesulphonic acid. When necessary the pH was adjusted to 3.4 with 1 M KOH or 1 M HCl. The separation of scopolamine and atropine using 30 % acetonitrile and 70 % buffered 0.01 M 1-heptanesulphonic acid is shown in Fig 3.1.3. The two alkaloids were clearly separated, and the peak shape was good, although a little asymmetric.

However, running the HPLC apparatus with mobile phases containing such high salt concentrations can be deleterious. Therefore the next mobile phase tested contained buffer at one tenth the concentration used above. The result, using 40 % MeCN, is shown in Fig 3.1.4, retention times were increased, but more importantly, the peak shape was poor with considerable tailing. The following factors were then manipulated in an attempt to improve peak shape:

the nature and concentration of the pairing ion
pH
the nature and concentration of the organic modifier
ionic strength
the nature of the solid phase (column packing material)

Changing the pairing ion to 1-hexanesulphonic acid, reduced the retention time of scopolamine by 0.45 min, and the peak shape improved. The effect of the concentration of the pairing ion (1-hexanesulphonic acid) on the retention time of atropine and scopolamine is shown in Fig 3.1.5. 1-hexanesulphonic acid was selected as the optimum type, and 0.015 M as the optimum concentration of

the pairing ion.

Reducing the pH of the buffer to 2.8 increased the retention of scopolamine, but increasing the pH to 4.0 and then to pH 4.6 also resulted in slightly longer retention times (Fig 3.1.6). The composition of the buffer at these different pH values is shown in Table 3.1.4. The mobile phase at pH 3.4 did give the shortest retention times, but the resolution of atropine and scopolamine was better at pH 4.0, which was therefore selected as the optimum mobile phase pH.

When methanol was used in place of acetonitrile as the organic modifier, the samples were retained on the column. The effect of the concentration of acetonitrile in the mobile phase on the retention times of both scopolamine and atropine is shown in Fig 3.1.7; 60 % acetonitrile was selected as the best concentration as it gave a reduced retention time and improved peak shape.

The effect of the ionic strength of the mobile phase has already been illustrated in the difference between Figs 3.1.3 and 3.1.4, where the more concentrated buffer gave a better peak shape and analysis time, but was not used in order to protect the HPLC apparatus.

The separation of scopolamine and atropine with 60 % acetonitrile and 40% 0.015 M 1-hexanesulphonic acid in dilute buffer at pH 4.0 (Table 3.1.4) is shown in Fig 3.1.8. It may have been possible to make further improvements in this mobile phase by fine adjustments of these parameters, but at this point the effect of the stationary phase was examined.

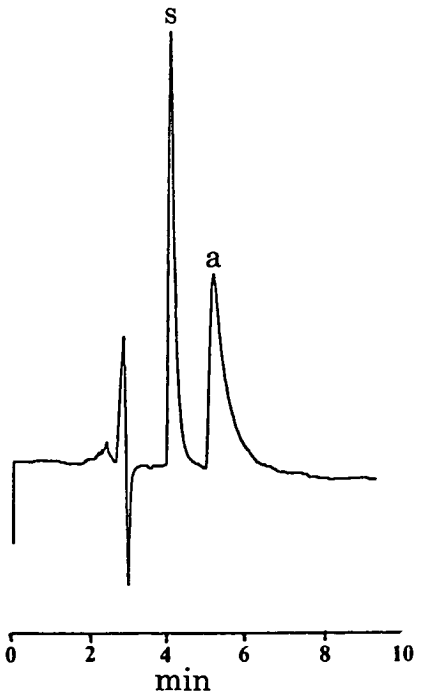


Fig 3.1.3

A chromatogram showing the separation of scopolamine (s) and atropine (a) by HPLC, using a C_{18} column, eluted at 1 $mlmin^{-1}$ with 30% MeCN and 70% 0.01 M heptanesulphonic acid in concentrated citric acid:Na_2HPO_4 buffer at pH 3.4, with detection at 254 nm.

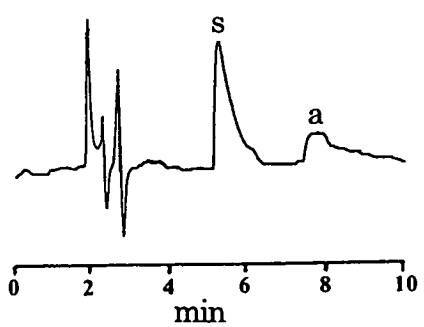
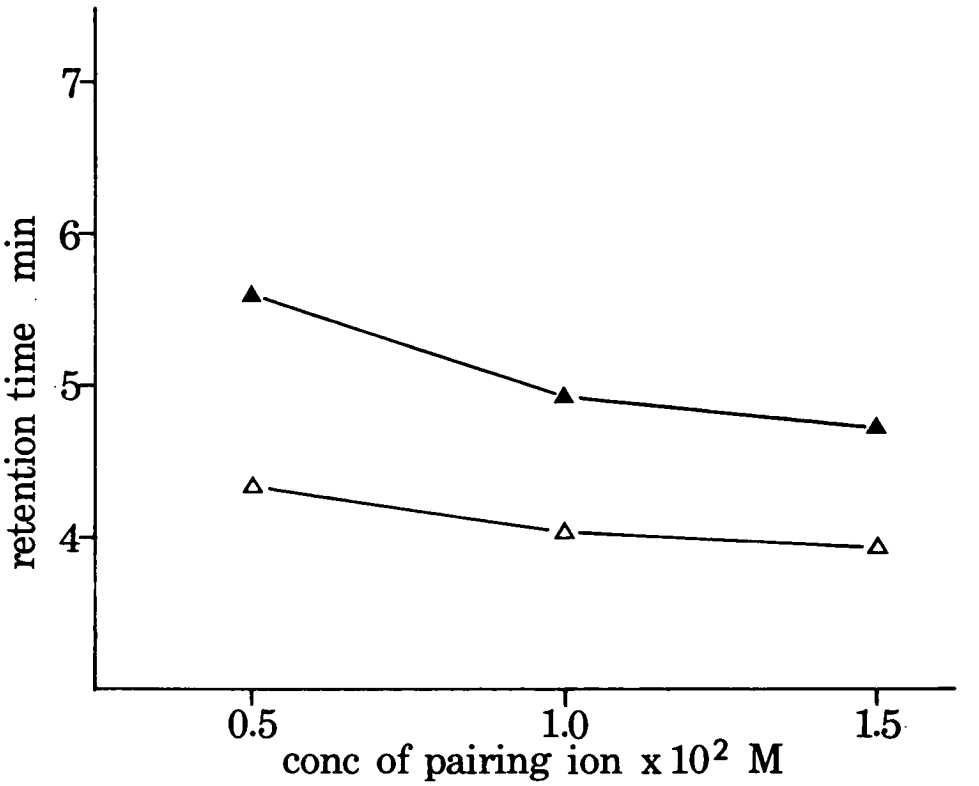


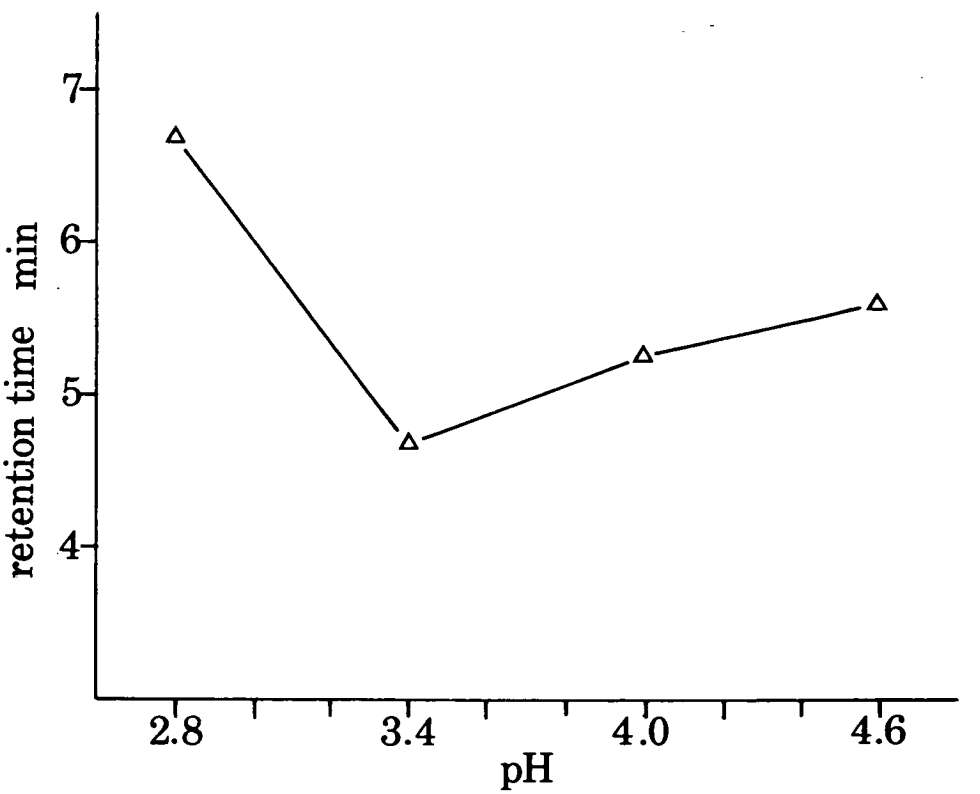
Fig 3.1.4

A chromatogram showing the separation by HPLC of scopolamine (s) and atropine (a) using a C_{18} column eluted at 1 $mlmin^{-1}$ with 40% MeCN and 60% 0.01 M heptane sulphonic acid in dilute citric acid:Na_2HPO_4 buffer at pH 3.4, with detection at 254 nm.

Fig 3.1.5
The effect of the pairing ion concentration on the retention of atropine (dark triangles) and scopolamine (open triangles) on a C_{18} column eluted with a mobile phase consisting of 60% MeCN and hexanesulphonic acid in citric acid:Na_2HPO_4 buffer at pH 4.6.

Fig 3.1.6
The effect of pH on the retention of scopolamine on a C_{18} column eluted with a mobile phase consisting of 40% MeCN and 60% 0.005 M hexanesulphonic acid in citric acid:Na_2HPO_4 buffer at pH 2.8 to 4.6.


retention time min
7
6
5
4
0.5
1.0
1.5
conc of pairing ion x 10² M


retention time min
7
6
5
4
2.8
3.4
4.0
4.6
pH

pH	conc. of citric acid $mmoll^{-1}$	conc. of Na_2HPO_4 $mmoll^{-1}$
2.8	1.768	0.450
3.4	1.502	0.809
4.0	1.291	1.095
4.6	1.119	1.328

Table 3.1.4
The constituents of the dilute citric acid:di-sodium hydrogen orthophosphate (Na_2HPO_4) buffer derived from Data for Biochemical Research (1969).

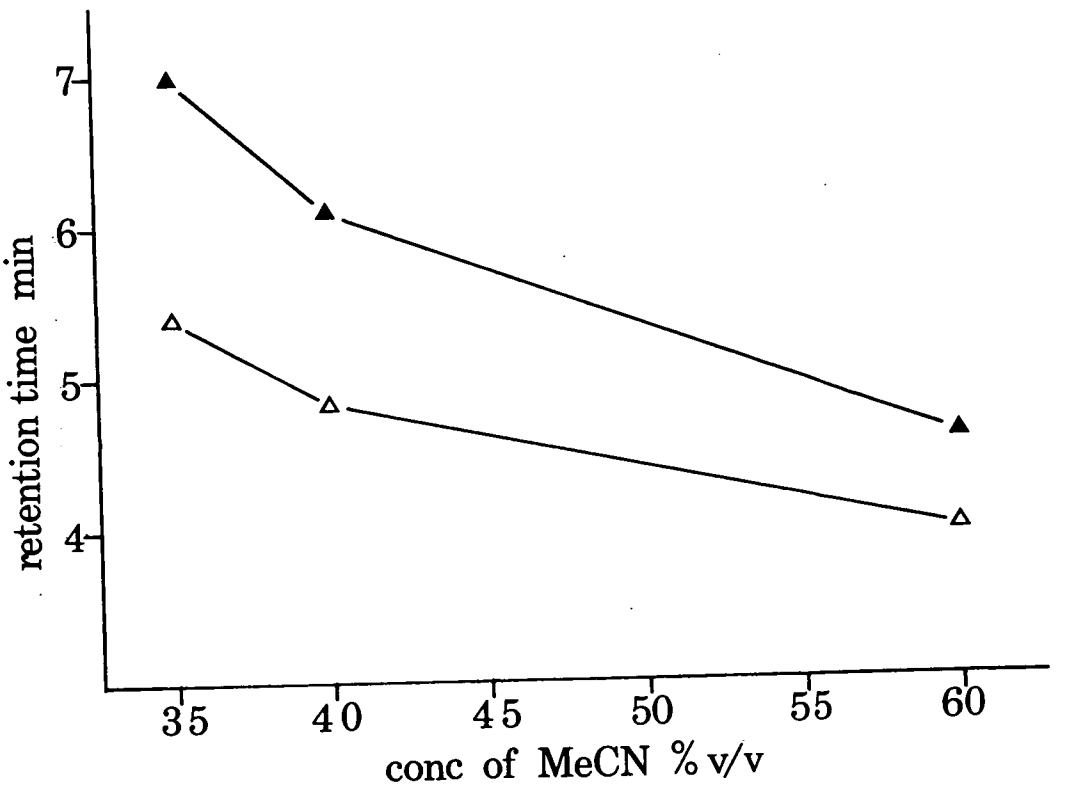


Fig 3.1.7
The effect on the retention time of atropine (dark triangles) and scopolamine (open triangles) on a C_{18} column of a mobile phase consisting of different proportions of MeCN and 0.01 M hexanesulphonic acid in citric acid:Na_2HPO_4 buffer at pH 4.0.

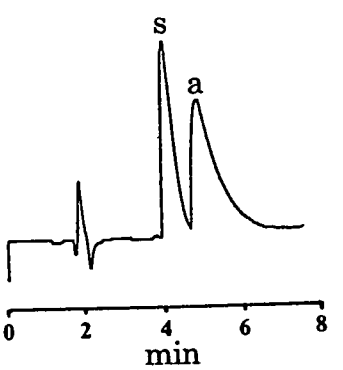


Fig 3.1.8
The separation of atropine (a) and scopolamine (s) by HPLC on a C_{18} column eluted at 1 $mlmin^{-1}$ with a mobile phase consisting of 60% MeCN and 40% 0.015 M hexanesulphonic acid in dilute citric acid:Na_2HPO_4 buffer at pH 4.0, with detection at 254 nm.

A 25cm x 4.6 mm (internal diameter) Octyl (C_8) column (Phase Separations - a gift which is gratefully acknowledged) was eluted with the modified mobile phase described above (composed of acetonitrile and 0.015 M 1- hexanesulphonic acid at pH 4.0). Samples of atropine, scopolamine and hyoscyamine-N-oxide were separated using 40 % acetonitrile as shown in Fig 3.1.9. The peak shape was good despite the longer retention times and the alkaloids were clearly resolved.

The mobile phase derived from Baumann's system (composed of acetonitrile and 50 mM KH_2PO_4 at pH 3.0), which had earlier been rejected (Section 3.1.2.1) was also tried with the Octyl column. The separation of alkaloids shown in Fig 3.1.10 was obtained at 25 % acetonitrile. This is an improvement on the separation shown in Fig 3.1.9, as the peaks were more symmetrical, and the analysis time was shorter. This method was therefore selected for the analysis of tissue extracts. The proportion of acetonitrile and aqueous buffer had to be modified slightly when complex tissue culture extracts were analysed. 22.5 % acetonitrile and 77.5 % buffer were used while the alkaloids were eluted, then the acetonitrile concentration was increased in order to elute the rest of the sample components more quickly and thus reduce the analysis time. These modifications are described in Section 2.4.3.3, together with the methods for identifying the alkaloid peaks from tissue extracts, and calculating the alkaloid concentration in the tissue.

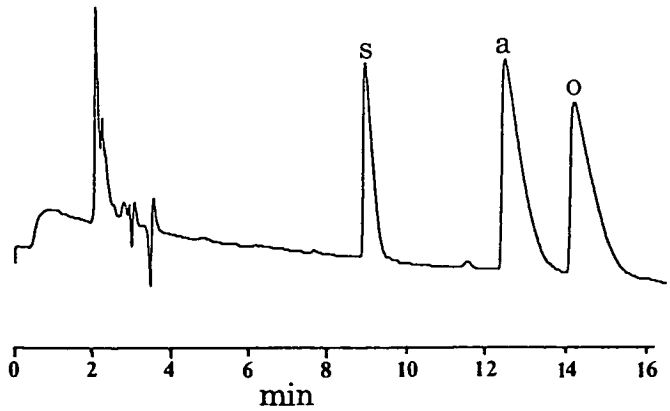


Fig 3.1.9

The separation of scopolamine (s), atropine (a) and hyoscyamine-N-oxide (o) by HPLC on a C_8 column eluted at 1 $mlmin^{-1}$ with a mobile phase consisting of 40% MeCN and 60% 0.015 M hexanesulphonic acid in dilute citric acid:Na_2HPO_4 buffer at pH 4.0, with detection at 254 nm.

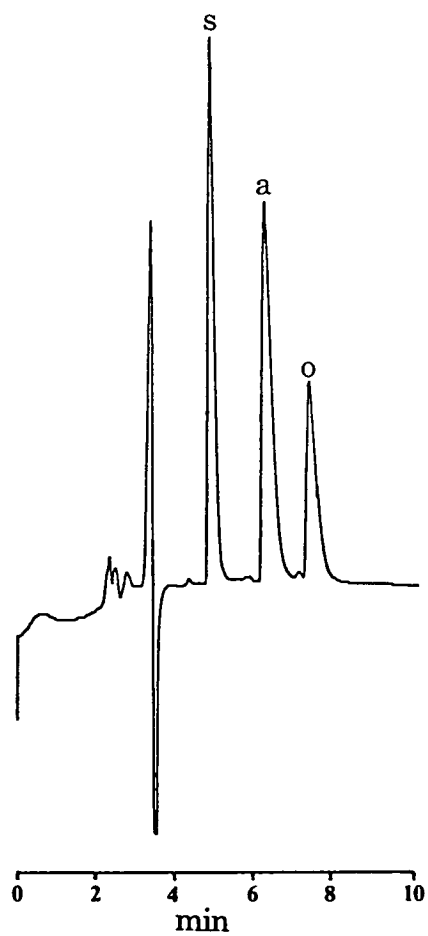


Fig 3.1.10

The separation of scopolamine (s), atropine (a) and hyoscyamine-N-oxide (o) by HPLC using a C_8 column eluted at 1 $mlmin^{-1}$ with a mobile phase consisting of 25% MeCN and 75% 50 mM KH_2PO_4 at pH 3.0, with detection at 245 nm.

Section 3.1.2.3 The Reasons for the Choice of HPLC as a Method of Analysis for Tropane Alkaloids

The criteria for the selection and development of a method for the determination of tropane alkaloids in plant cell cultures were; sensitivity, accuracy, good separation, and speed.

The Vitali Morin colorimetric test and TLC are relatively fast methods but they cannot satisfy the two more important criteria, resolution and sensitivity, and are not specific to tropane alkaloids. The Vitali Morin test (Roberts & James 1947) can be used to determine the total alkaloid content of extracts, or to determine the amount of a single alkaloid separated by TLC. But 0.05-0.15 mg of alkaloid is required for the test, and such amounts were seldom present in the cell culture extracts. Furthermore, although a strong colour reaction is only produced by esters of tropic and truxillic acids, other compounds which contain benzene rings can cause weak reactions (James & Roberts 1945).

The TLC solvent systems described in Section 2.4.2 clearly resolved standard samples of hyoscyamine, hyoscyamine-N-oxide, scopolamine, and tropine. However, these were not clearly separated from other "Dragendorff positive substances" which were present in cell culture extracts. Most of these "Dragendorff positive substances" were not considered to be tropane alkaloids because i) they did not react with Iodoplatinate reagent; ii) extracts which contained large amounts of such substances did not produce a colour reaction with the Vitali Morin test; iii) Dragendorff's reagent can react with a wide range of compounds, the minimum structural features necessary being conjugated ketone or aldehyde groups (Farnsworth *et al* 1962).

The HPLC method described in Section 3.1.2 satisfies the most important criteria, separation and sensitivity (1 µg of atropine can be determined); and the speed and ease of operation are at least equivalent to that of TLC and the Vitali Morin test, (if breakdowns of the apparatus are ignored). The internal standard method, which was used to confirm the identity of the atropine peak in at least one from each group of extracts, makes this method of analysis relatively specific, and the presence of atropine could also be checked by spectral analysis of the peak using the Hewlett Packard 1090 HPLC.

Other analytical methods have certain advantages over HPLC. Immunological techniques, such as the radioimmunoassay developed by Lehtola *et*

al (1982), are very sensitive and highly specific. However, in addition to the disadvantage of the considerable amount of time needed to isolate a good antibody preparation, the method only determines one alkaloid, and there is a possibility of cross reaction.. Gas Liquid Chromatography (GLC) and Mass Spectrophotometry (MS) are powerful methods for the separation and accurate identification of a wide range of alkaloids, however Oprach *et al* (1986) who used GLC/MS to determine a range of alkaloids in extracts from *A. belladonna* found that TLC and HPLC were also needed in order to identify the N-oxides. Furthermore GLC is time consuming and requires derivatization of the alkaloids, and MS requires considerable time and effort even in the hands of an expert. Therefore HPLC is considered to be the most suitable method for the routine analyses of this study.

Section 3.2 THE DEVELOPMENT AND CHARACTERIZATION OF THE IMMOBILIZATION PROCEDURE FOR CULTURED CELLS OF *ATROPA BELLADONNA* AND *HYOSCYAMUS MUTICUS*

The aim of the experiments described in this section was to develop a procedure for the immobilization of cells of *A. belladonna* and *H. muticus*. In the method chosen for this investigation, polyurethane foam blocks were added to a freshly subcultured cell suspension culture. The cells then became trapped in the foam, where they grew and divided until the blocks were packed with cells. This procedure has been described in detail in Section 2.2.4.

The first experiment described in this section was performed to find out if cells of *A. belladonna* could be immobilized in polyurethane foam, and to study their growth and viability during this process. Having established that cells of *A. belladonna* were immobilized by this method, the next experiment was performed to define more precisely their growth during immobilization, and to find the optimum conditions in terms of the maximum immobilized biomass that could be obtained from a specified size of inoculum. After this the method was judged to be satisfactory and it was not considered necessary to make any further changes in the immobilization procedure for cells of *A. belladonna*. The last part of this section describes an experiment performed to find out if the same method could also be used to immobilize cells of *H. muticus*, and, as suspension cultures of *H. muticus* were coarser than those of *A. belladonna*, the effect of the foam pore size on immobilization was also examined.

Section 3.2.1 A PRELIMINARY EXPERIMENT TO IMMOBILIZE SUSPENDED CELLS OF *ATROPA BELLADONNA* IN POLYURETHANE FOAM

The aim of this experiment was to find out if suspended cells of *Atropa belladonna* could be immobilized in polyurethane foam, and to examine and characterize their growth during immobilization. The growth of both suspended and immobilized cells within each culture flask was monitored during the culture period by determining their fresh and dry weights, cell number, and cell viability.

At time 0, 5 ml aliquots taken from ten stock suspension cultures (grown in SH medium), were added aseptically to 50 ml of SH medium in twenty eight 250 ml culture flasks. After thorough mixing, 5 ml was removed from each flask to determine the original cell number and assess the cell viability. The mean

population density at time 0 was 3.42 x 10^6 +/- s.e. 5.34 x 10^5 cells per flask. After inoculation, four foam blocks were added to each of the flasks which were then incubated on a rotary shaker under the conditions described in Section 2.2.3.1.

Four flasks, taken at random, were harvested on days 3, 5, 6, 7, 10, 12, and 14. The f. wt., dry wt., and cell number of both the suspended and the immobilized cells in each flask were determined as described in Section 2.3.1. These results are presented in Figs. 3.2.1, 3.2.2, and 3.2.3. Cell viability was also determined using fluorescein diacetate but the results have not been presented here as the method was found to be unsuitable for cells of *A. belladonna*. The stain did not produce the two clearly distinguishable populations expected, i.e. brightly fluorescent (viable) and non-fluorescent (non-viable). Instead, most cells exhibited an overall fluorescence, always relatively faint, but varying in intensity, thus it was difficult both to define and to distinguish "viable" cells, and therefore it was not possible to form any useful conclusions from such data.

Cell Number (Fig 3.2.1)
During the first five days, there was a decrease in the suspended and the total cell number. The decrease in the total cell number was probably due to cell death. On day 5, the total cell number was greater than the suspended cell number. This difference can be accounted for by some cells becoming trapped and immobilized in the foam blocks. The suspended cell population increased between days 5 and 6. The immobilized cell number did not increase until later, between days 6 and 7, then remained relatively constant until the end of the culture period.

Fresh and Dry Weights (Figs 3.2.2 and 3.2.3)
During the first five days, there was a decrease in the biomass of the total and the suspended cells. The decrease in the suspended cell weight was greater, which can again be accounted for by cell immobilization. It is not possible from the fresh weight data to distinguish when the cells began to grow, as the variation between replicates was too great. However, the dry weight data indicate that the suspended cell biomass increased between days 6 and 7, and appears to have decreased slightly towards the end of the culture period.

The standard errors in these results are very large. Variation in the nature and size of the inoculum probably contributed to this. Ten stock suspension

cultures were used as sources of inoculum, they were apparently uniform, but could have had different growth rates or responses to the immobilization procedure. Perhaps more important than any qualitative difference which may have existed between inocula was the variation in the inoculum size (mean 3.42 x 10^6 +/- s.e. 5.34 x 10^5 cells per flask). This could have affected the final culture weight, and the time when the cells began and ceased to grow. Variation in the inoculum size was due partly to the differences in population density which must have existed between the stock suspension cultures. However, the inoculum size varied chiefly because the stock cultures were transferred with a pipette. These stock cultures were fine, but dense, and so tended to block the pipette, resulting in inaccuracy. This method was not used for the inoculation of cultures in future experiments. In the next two experiments, aliquots of stock cultures were measured using sterile, graduated, Universal tubes (Sterilin).

The final culture weight reached in this experiment, was less than expected (see Section 3.2.2). This could be because these cultures were relatively newly initiated, and with repeated subculture, faster growing cells would have been selected.

Despite the variation in the results, it is evident that cells of *A. belladonna* can be immobilized in polyurethane foam. The next experiment describes an attempt to optimize the immobilization procedure, and to further characterise growth during immobilization.

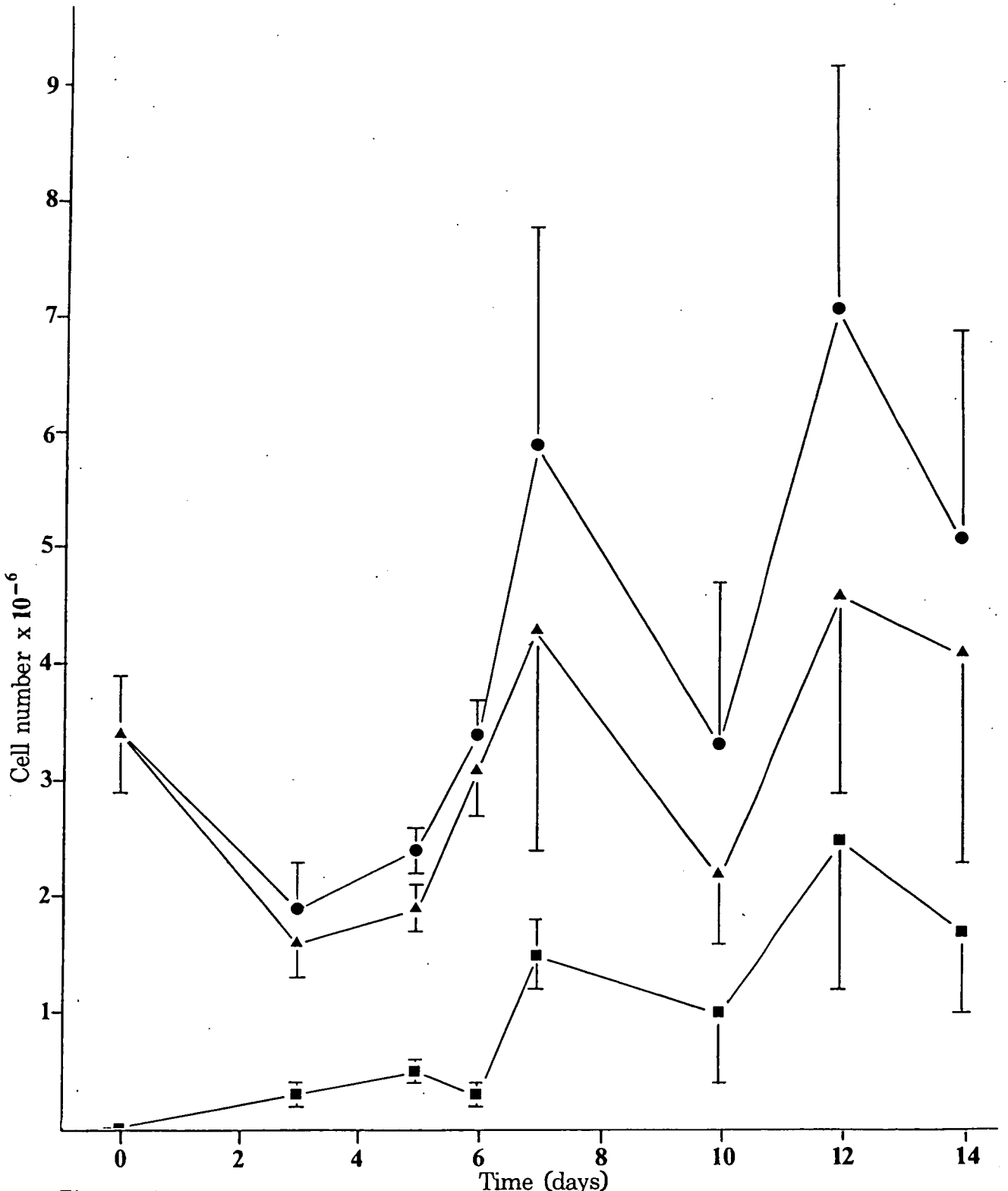


Figure 3.2.1

Changes in the immobilized (squares), suspended (triangles), and total (circles) cell number during the immobilization of *A. belladonna* cells in polyurethane foam (each value is the mean of four replicates +/- s.e.)

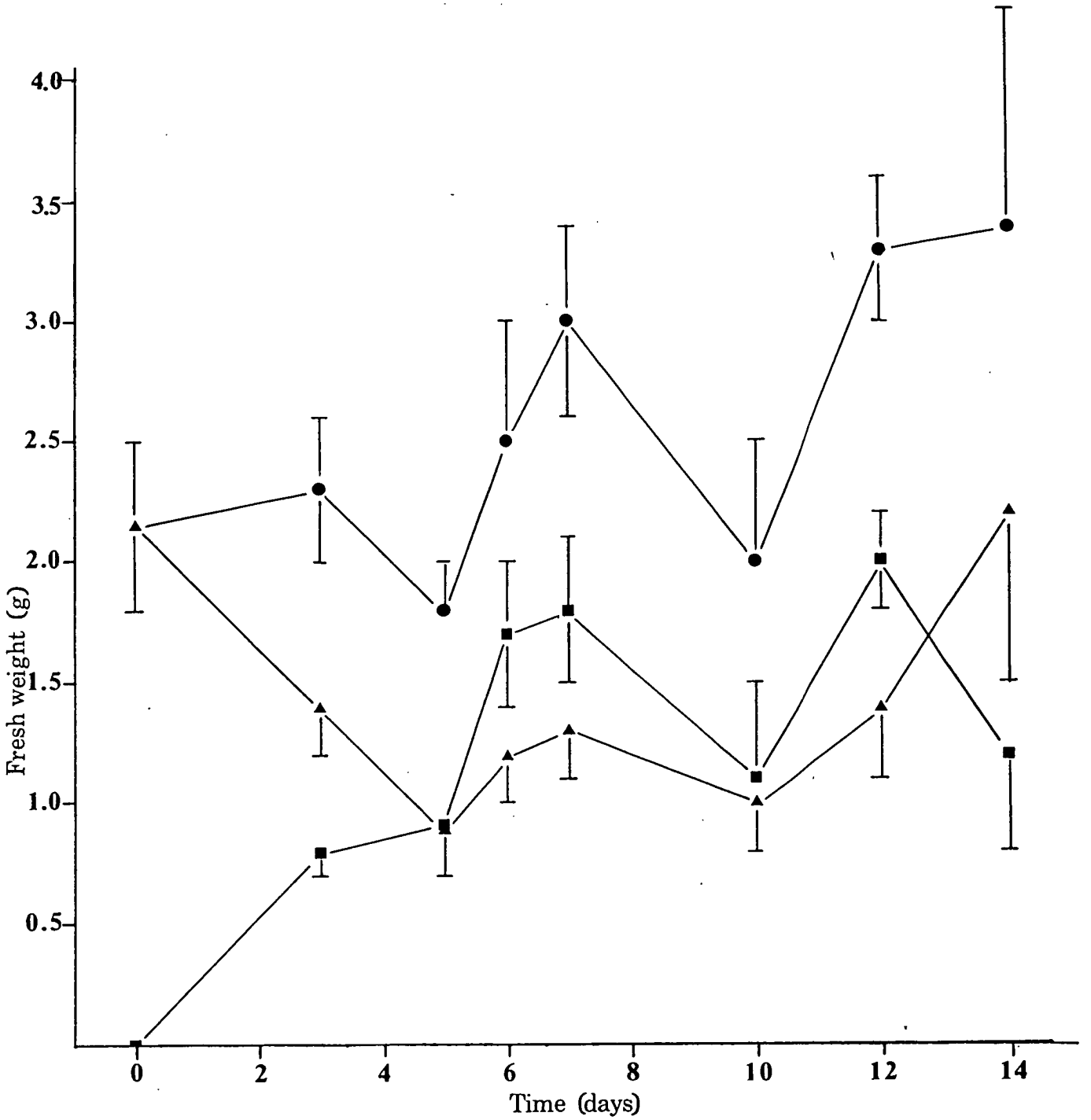


Figure 3.2.2

Changes in the fresh weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in polyurethane foam (each value being the mean of four replicates +/- s.e.)

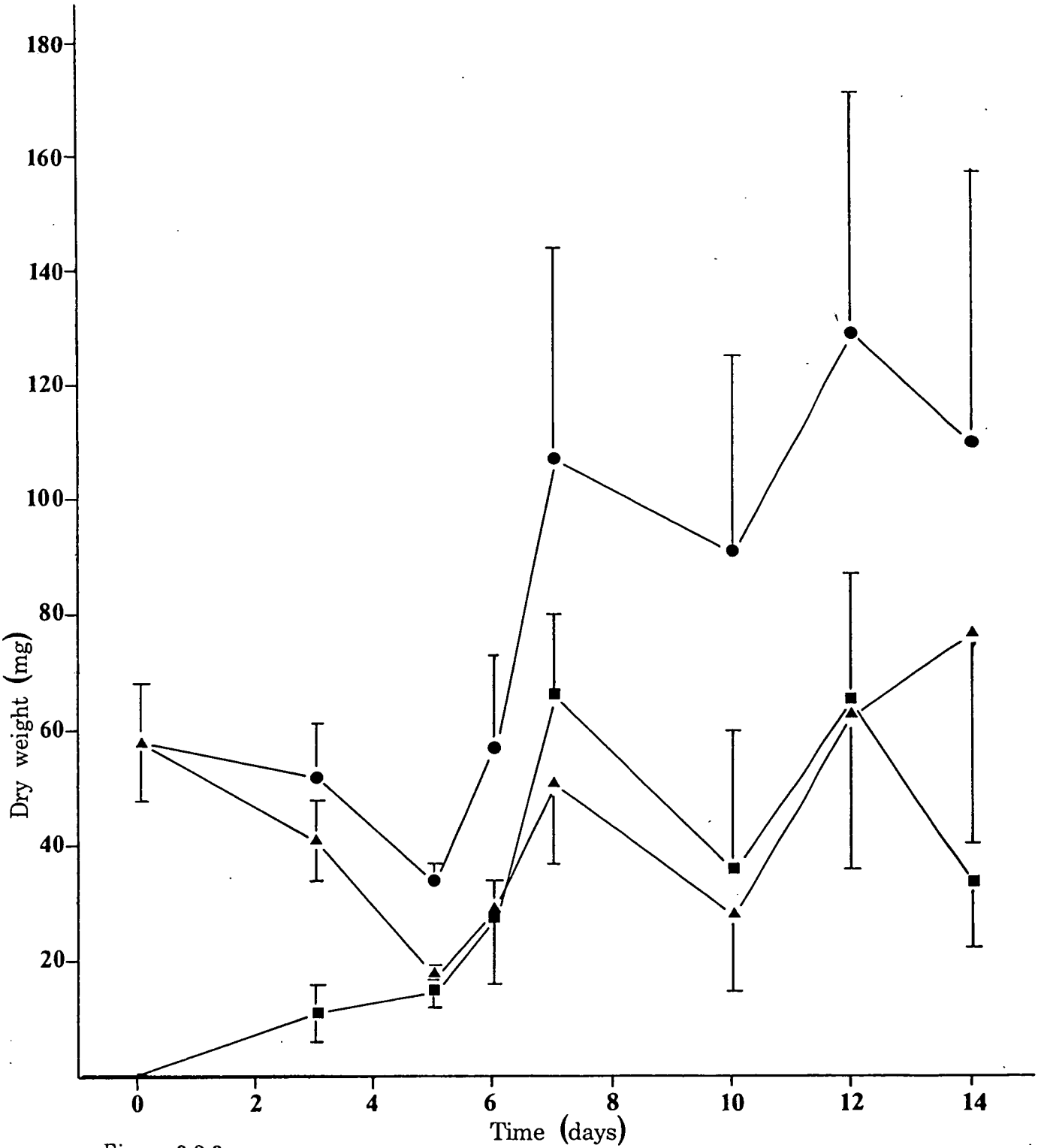


Figure 3.2.3

Changes in the dry weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in polyurethane foam (each value being the mean of four replicates +/- s.e.)

Section 3.2.2 DETERMINATION OF THE OPTIMUM NUMBER OF FOAM PARTICLES FOR THE IMMOBILIZATION OF CELLS OF *ATROPA BELLADONNA*

The levels of tropane alkaloids previously found in unorganized cell cultures of *A. belladonna* have usually been low (see Table 1.1) compared to those in the plant, 3 mgg^{-1} dry wt. in leaves 4 mgg^{-1} in roots (Merck Index 1983). Therefore in order to study tropane alkaloid production by immobilized cells, large amounts of immobilized biomass were required. This experiment was an attempt to increase the immobilized biomass that could be obtained from a specific size of inoculum.

On day 0, 15 ml aliquots taken from twenty stock suspension cultures were added to 50 ml of SH medium in 48 culture flasks, using sterile, graduated, 25 ml Universal tubes (Sterilin). The mean f. wt. of the inocula was 2.501 +/- s.e. 0.41 g per flask. The flasks were divided into three groups of 16. Four foam blocks were added to each flask in one group, seven and ten blocks to each flask in the other groups, before they were incubated on a rotary shaker. On days 3, 8, 12, and 16, three flasks, and on day 21, four flasks from each group were harvested, and the fresh and dry weights of the immobilised and suspended cells were determined (Section 2.3.1). The results are presented in Figs. 3.2.4 to 3.2.9.

The biomass of the suspended cells and of the whole cultures did not increase during the first three days, but there was no loss of biomass as in the last experiment. After day 3, the total biomass of all the cultures showed a similar increase, until day 8 in cultures containing seven blocks, and until day 12 in the other treatments. The total dry wt. then remained the same, but the f. wt. continued to increase in the cultures containing four and seven blocks until day 21.

The dry wt. of suspended cells in flasks containing four and ten blocks increased until day 12, but only until day 8 in those containing seven blocks. The f. wt. of suspended cells levelled off when the dry wt. stopped increasing, but in flasks containing four and seven blocks, it began to increase again, after day 16, and this continued until the end of the culture period. The final dry wt. of suspended cells was similar in the cultures containing four and seven blocks, but was significantly less in cultures containing ten foam blocks.

The pattern of growth of the immobilized cells was similar in all the

treatments in terms of their dry wt.. It increased from time 0 until day 8 in seven blocks, and until day 12 in four and ten blocks. The f. wt. of immobilized cells also remained stable after day 12 in the latter two treatments, but in seven foam blocks, it levelled off until day 12, then began to increase again until the end of the culture period.

Using ten foam blocks increased the immobilized biomass that could be obtained from the original inoculum. Therefore ten blocks were used to immobilize cells in the experiment described in Section 3.3.

No further experiments were performed to optimize the immobilization procedure for *A. belladonna*, as it now yielded the maximum possible number of densely packed foam particles. The next section describes an experiment to examine the effect of foam pore size on the immobilization of cells of *H. muticus*.

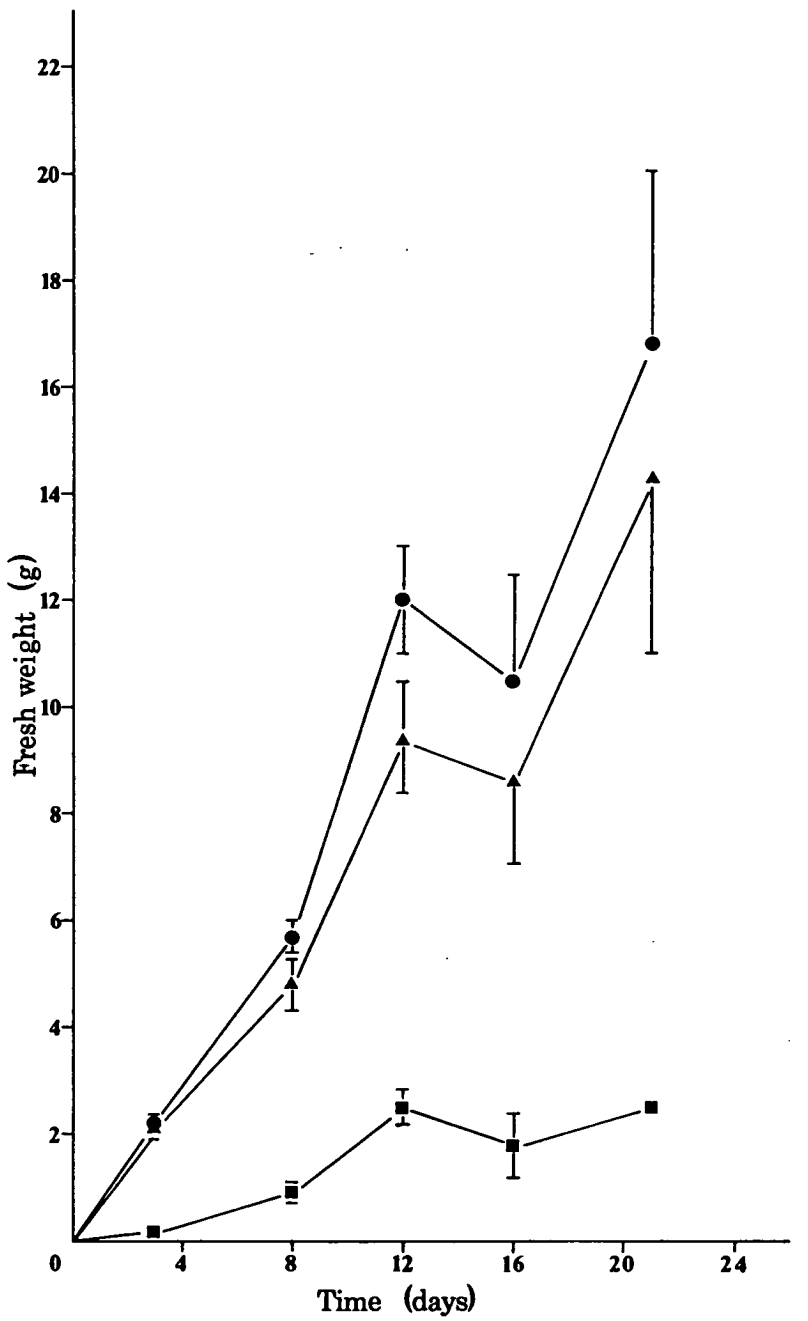


Figure 3.2.4

Changes in the fresh weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in four foam blocks (each value is the mean of three replicates +/- s.e.)

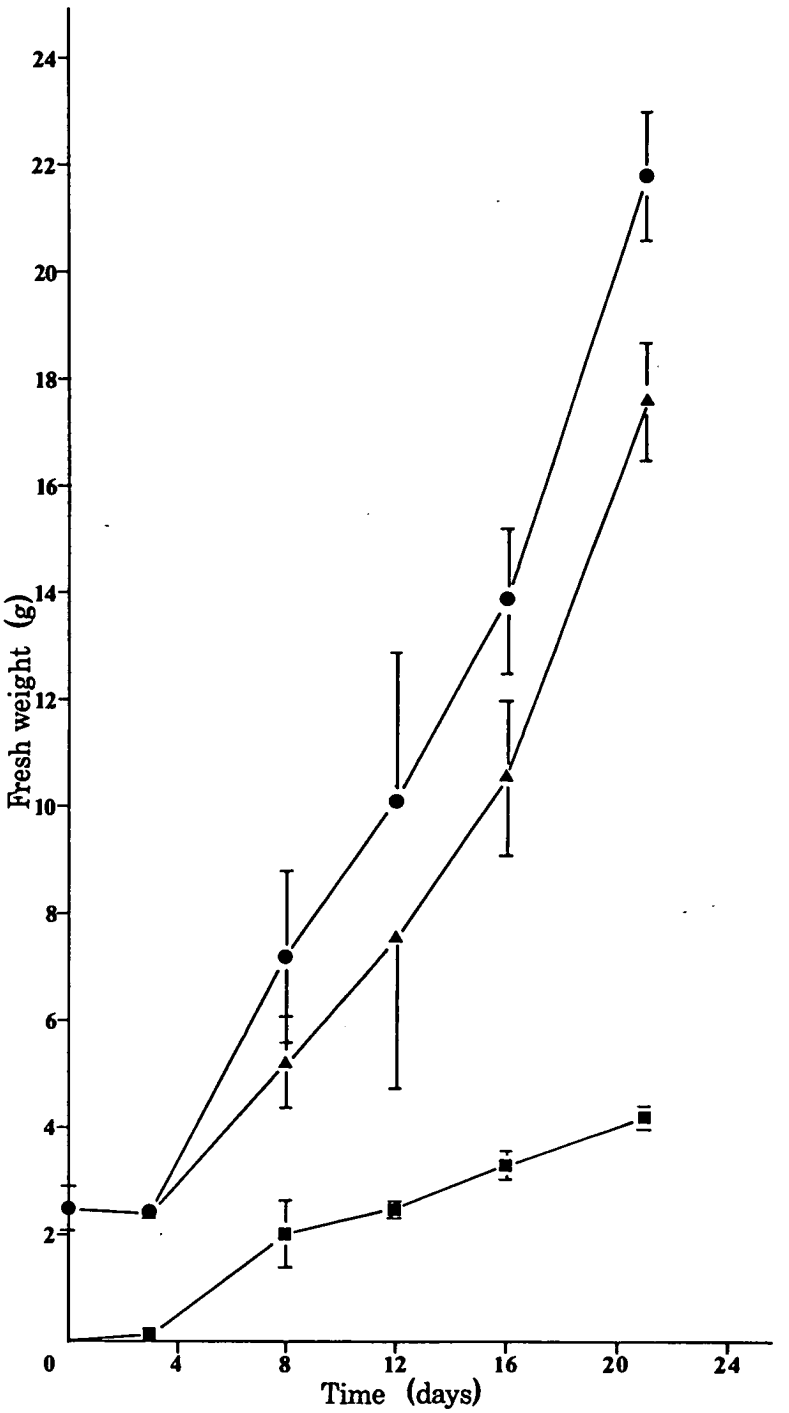


Figure 3.2.5

Changes in the fresh weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in seven foam blocks (each value is the mean of three replicates +/- s.e.)

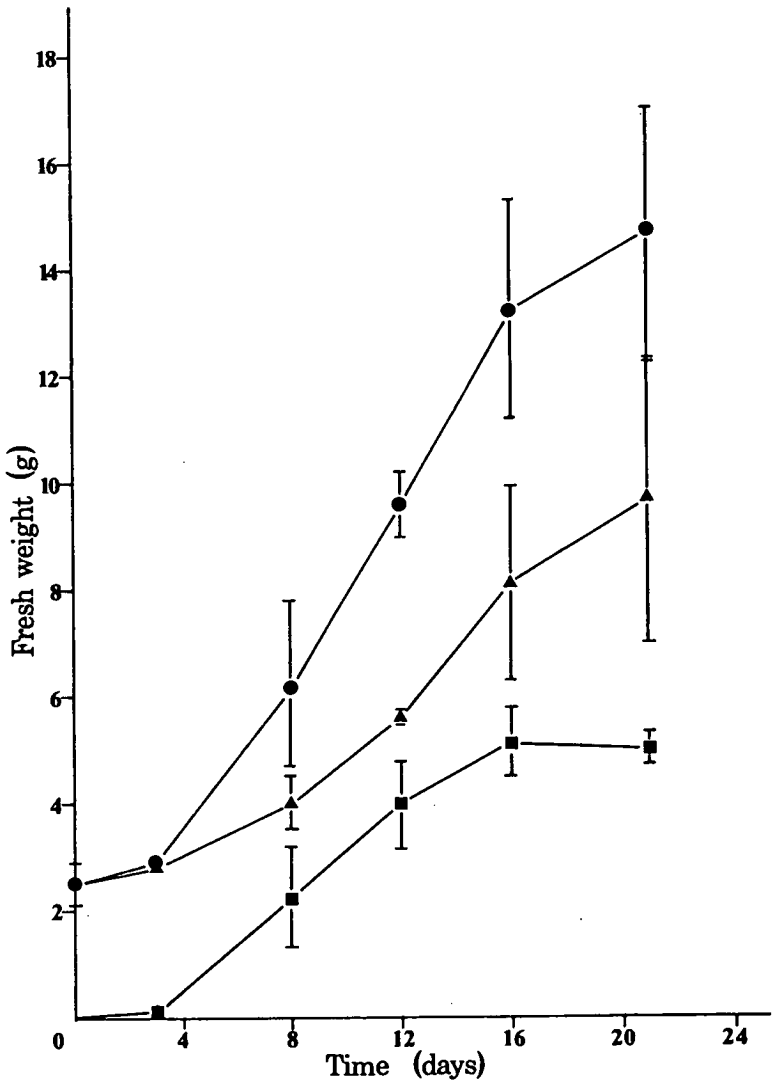


Figure 3.2.6

Changes in the fresh weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in ten foam blocks (each value is the mean of three replicates +/- s.e.)

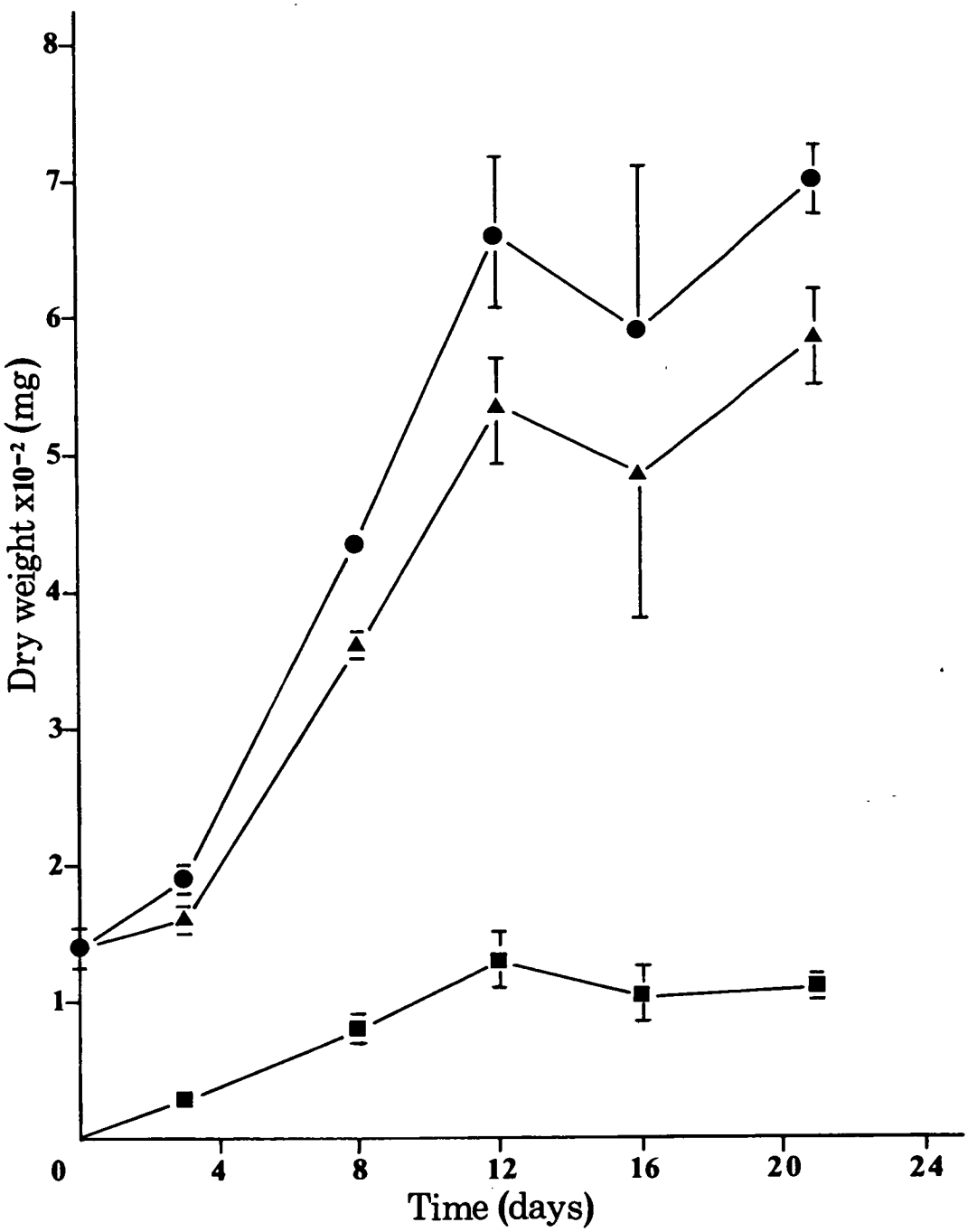


Figure 3.2.7

Changes in the dry weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in four foam blocks (each value is the mean of three replicates +/- s.e.)

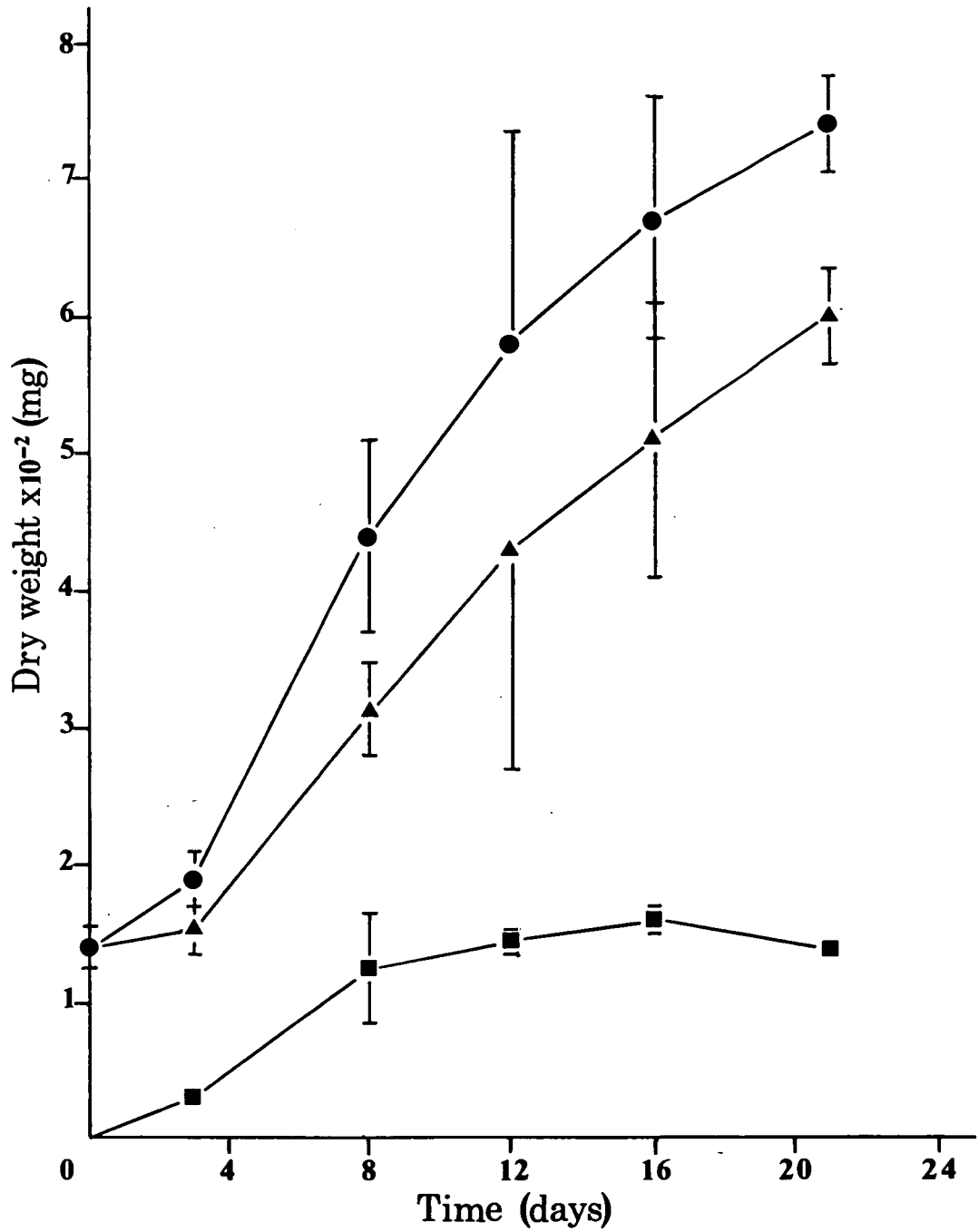


Figure 3.2.8
Changes in the dry weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in seven foam blocks (each value is the mean of three replicates +/- s.e.)

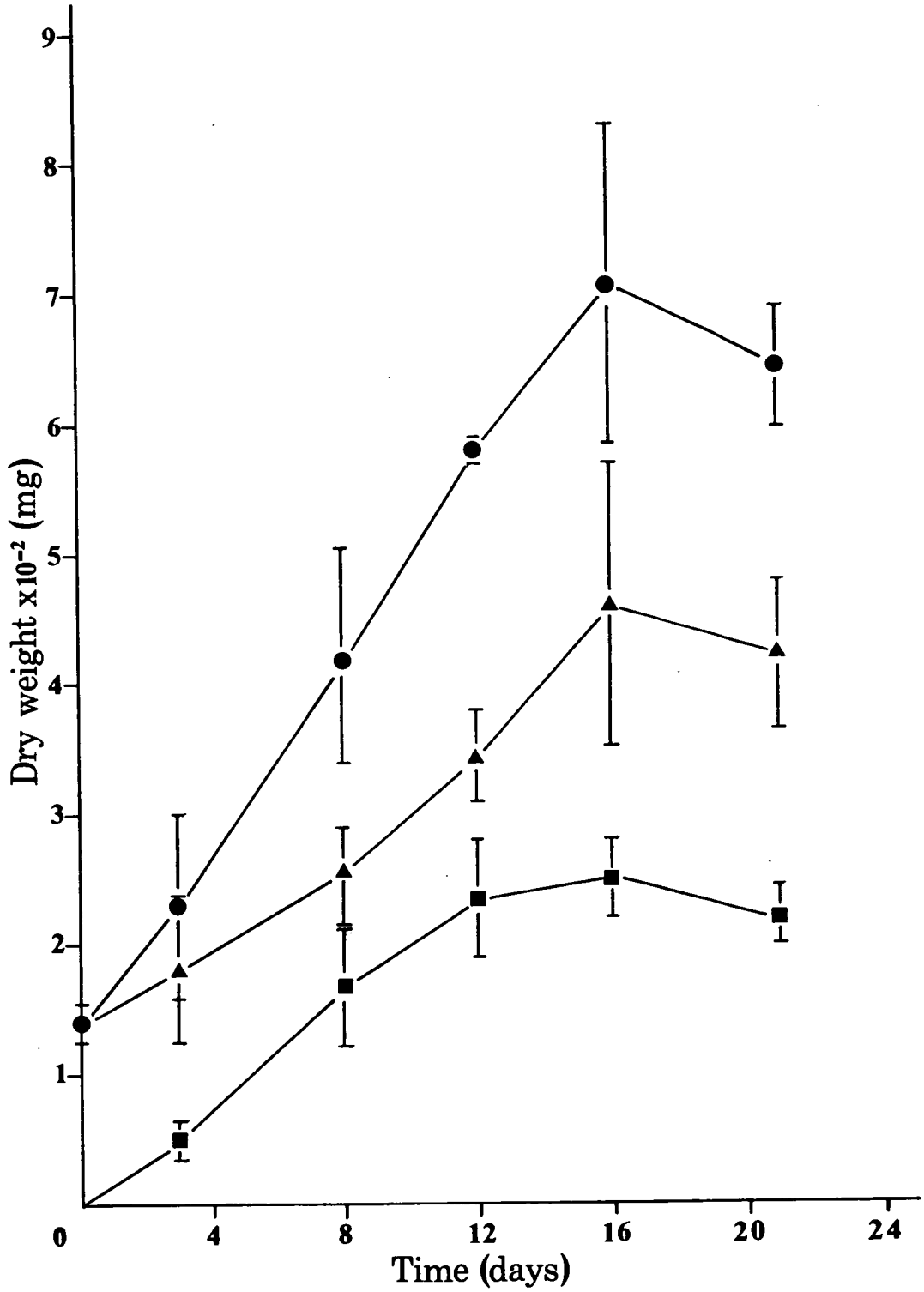


Figure 3.2.9

Changes in the dry weight of immobilized (squares), suspended (triangles), and total (circles) cells of *A. belladonna* during immobilization in ten foam blocks (each value is the mean of three replicates +/- s.e.)

Section 3.2.3 DETERMINATION OF THE OPTIMUM FOAM PORE SIZE FOR THE IMMOBILIZATION OF CELLS OF *HYOSCYAMUS MUTICUS*

Suspension cultures of *H. muticus*, especially when first initiated, are coarser than those of *A. belladonna*. Therefore the aggregates may be too large to enter the pores of the foam used to immobilize cells of *A. belladonna* (pore size = 40 pores per inch, ppi). In this experiment the efficiency of immobilization of cells of *H. muticus* in foam of two pore sizes (20 and 40 ppi) was compared.

At time 0, 20 ml aliquots taken from twenty stock suspension cultures were added to 30 ml of SH medium in forty eight 250 ml culture flasks, using sterile, graduated, 25 ml Universal tubes (Sterilin). The mean f. wt. of the inocula was 1.953 +/- s.e. 0.014 g per flask. Four foam blocks were added to each flask before they were incubated on a rotary shaker. Foam with a porosity of 20 ppi was added to 24 of the flasks, and foam with a porosity of 40 ppi was added to the rest. On days 3, 5, 7, 9, 11, and 14 three flasks containing foam of each pore size were harvested, and f. wt., dry wt. and cell number were determined (Section 2.3.1). The results are shown in Figs.3.2.10 to 3.2.12.

The total biomass (Figs 3.2.10 and 3.2.11) of these cultures had increased significantly by day 3, and continued to increase in a similar manner in both the treatments until day 11. After this point it continued to increase in the flasks containing foam with large pores (20 ppi), but did not in those containing foam with small pores (40 ppi).

The total cell number (Fig 3.2.12) increased in a stepwise manner, rising between days 3 and 5, and again, more slowly, between days 7 and 11. Again, the cultures containing 20 ppi foam grew until day 14, and those containing 40 ppi foam seem to have stopped growing by day 11.

The observed differences between treatments in the total biomass accumulated and the cell number at the end of the culture period are similar to differences in the growth of suspended cells, but not in the growth of immobilized cells, i.e. the suspended cells continued to grow until the end of the culture period in the presence of 20 ppi foam, but stopped growing after day 12 in cultures containing 40 ppi foam. The variation between replicates, particularly in terms of dry wt. and cell number, of the suspended cells is too great to distinguish clearly when growth began, but the data suggest that they did not begin to grow until relatively late in the culture period.

The immobilized cells in the blocks increased in number by approximately the same amount in both treatments from day 0 until day 5. Thereafter no further increase in cell number was observed in the 20 ppi foam, but after day 9, there was a further small increase in cell number in the 40 ppi foam (Fig 3.2.12). The immobilized biomass increased in a stepwise manner, first from time 0 until approximately day 7, then between days 9 and 11, after which there was a small decrease in the dry wt. of cells in the 20 ppi foam, but not in the 40 ppi foam. However there was no significant difference in the final dry wt. of the immobilized cells between the two treatments.

Therefore with the range of sizes used, pore size had no significant effect on the amount of immobilized biomass obtained. This was probably because the stock suspensions of *H. muticus* became more friable with repeated subculture, and because they were composed of aggregates of different sizes, some of which were small enough to enter the 40 ppi foam pores. To facilitate comparisons between immobilized cells of *A. belladonna* and *H. muticus*, both species were immobilized in foam of the same pore size (40 ppi), in all the following experiments.

In the next experiments the accumulation of tropane alkaloids in immobilized cells of *A. belladonna* and *H. muticus* was compared, and the production of alkaloids during the culture period was examined in the species which produced the higher level.

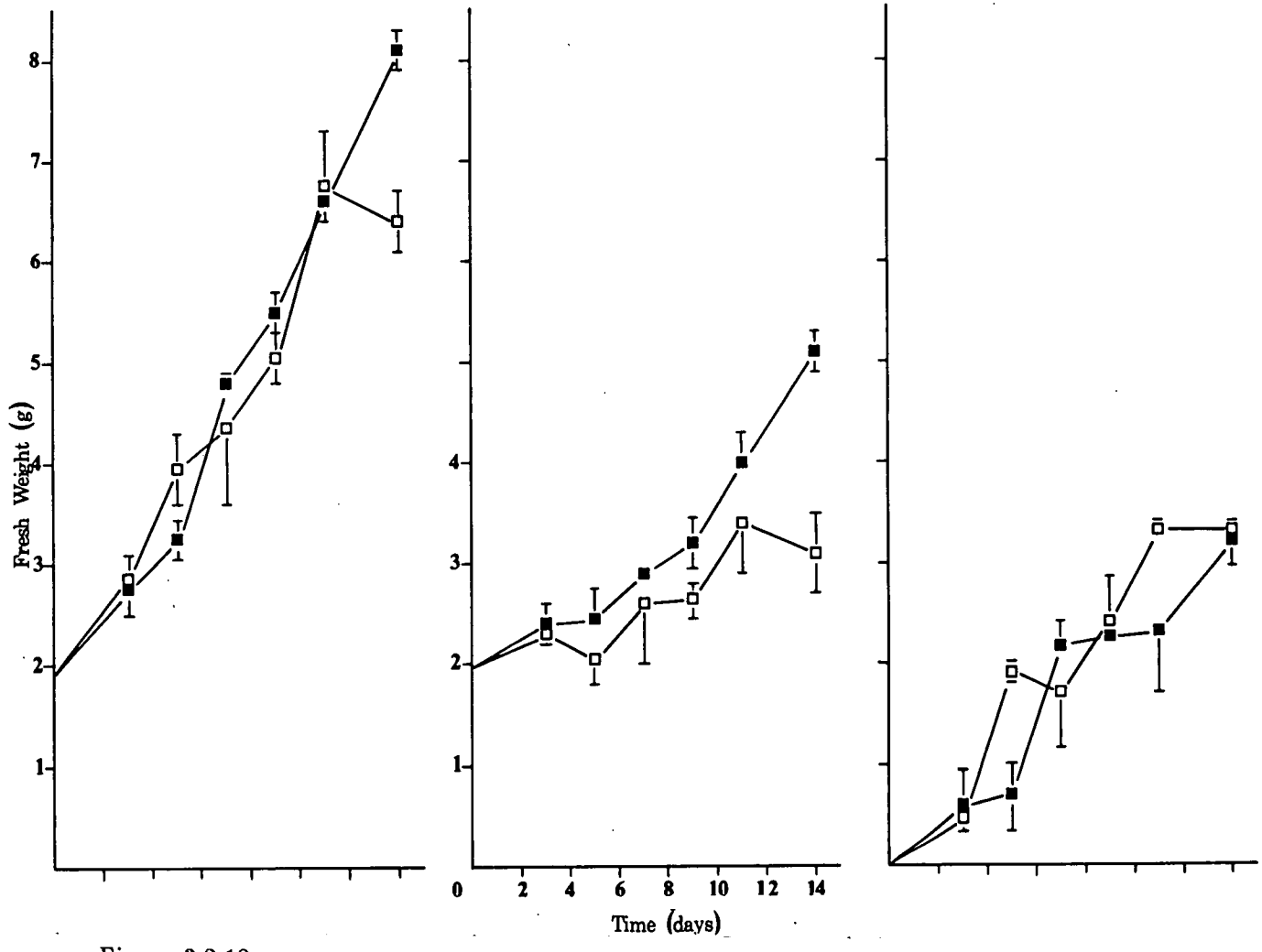


Figure 3.2.10

Changes in the fresh weight of total (left), suspended (centre), and immobilized (right) cells of *H. muticus* during immobilization in foam of two pore sizes, 20ppi (filled squares) and 40ppi (open squares). Each value is the mean of three replicates +/- s.e..

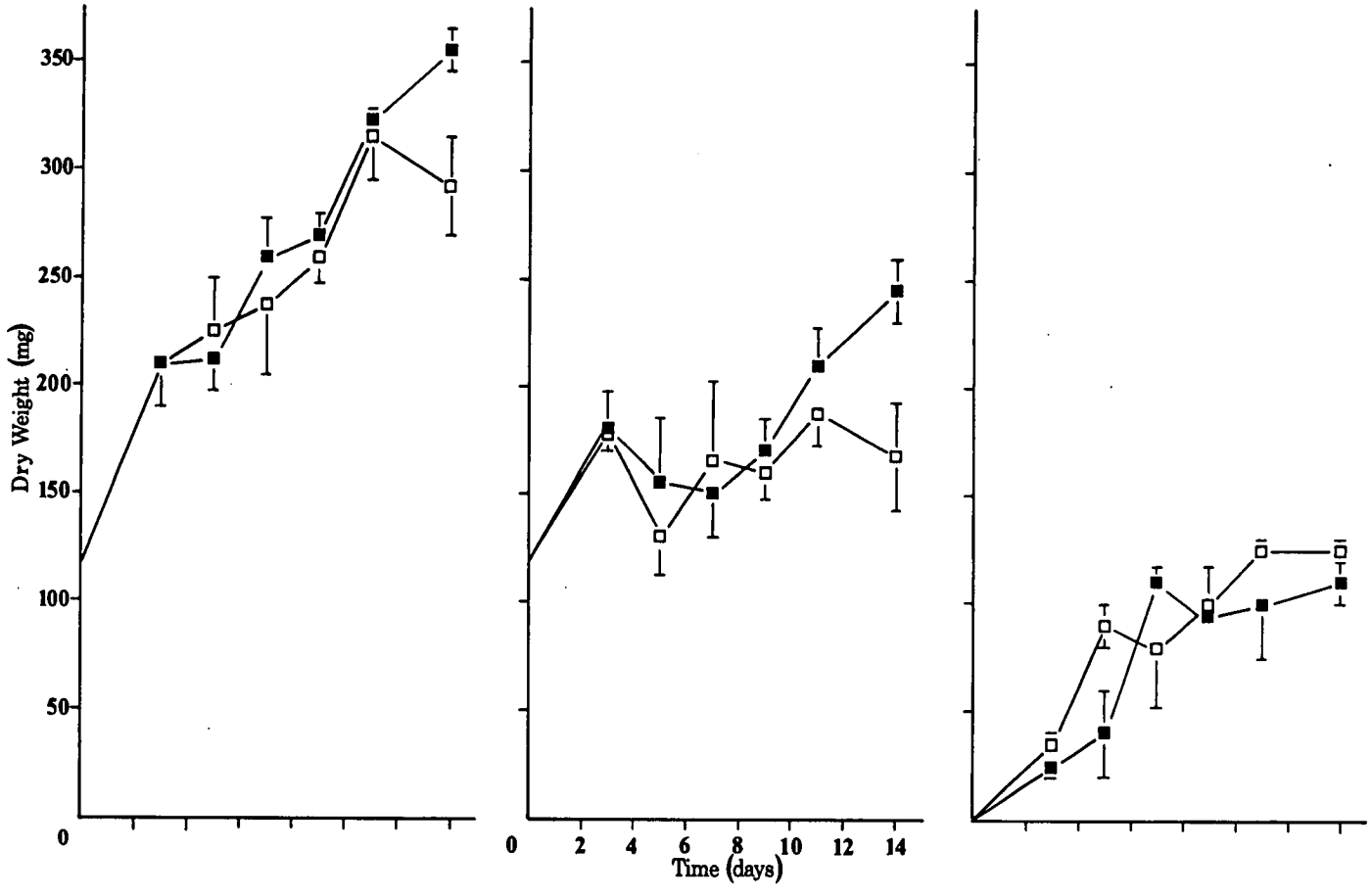


Figure 3.2.11

Changes in the dry weight of total (left), suspended (centre), and immobilized (right) cells of *H. muticus* during immobilizion in foam of two pore sizes, 20ppi (filled squares) and 40ppi (open squares). Each value is the mean of three replicates +/- s.e..

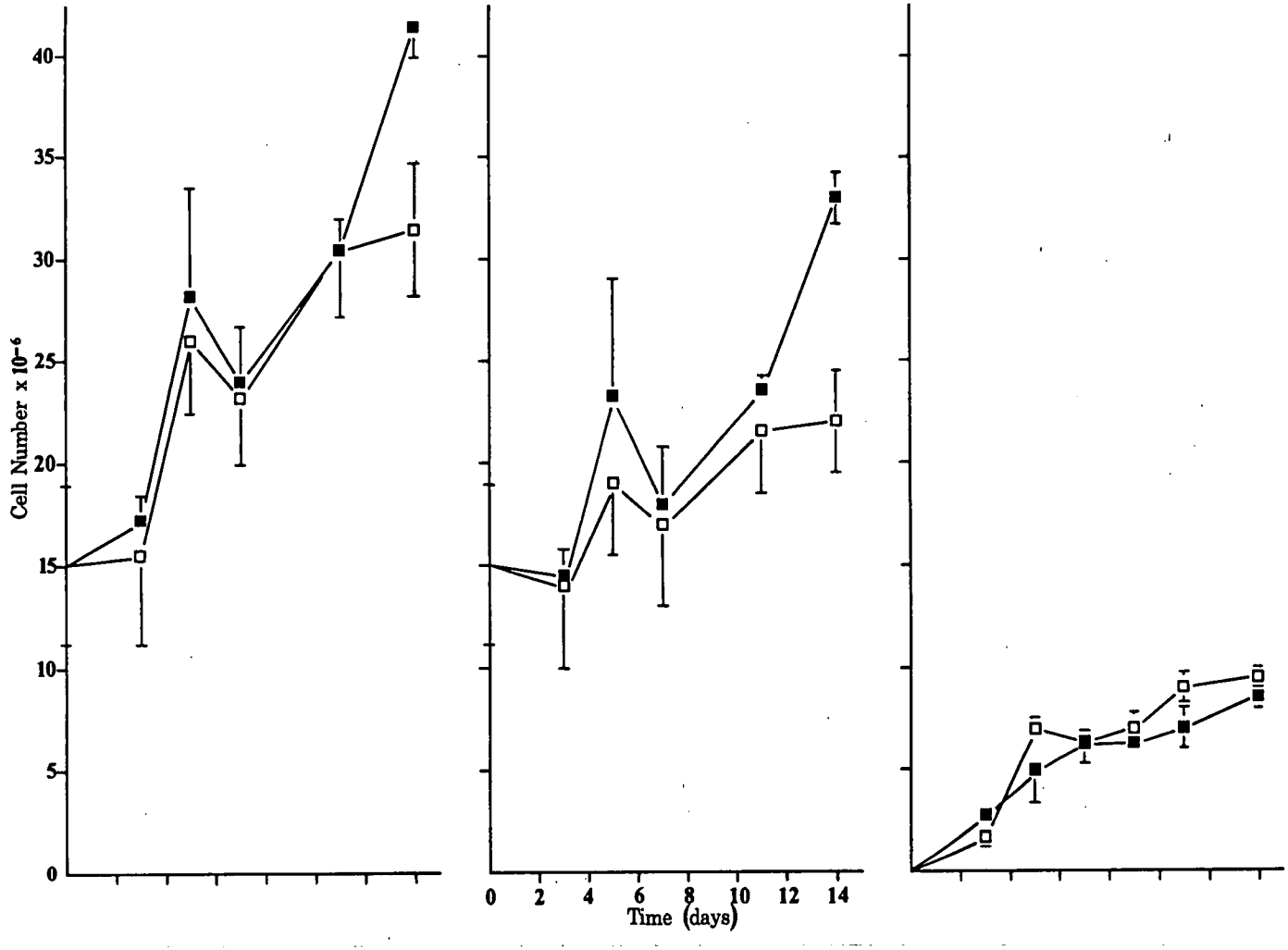


Figure 3.2.12

Changes in the total (left), suspended (centre), and immobilized (right) cell number in cultures of *H. muticus* during immobilizion in foam of two pore sizes, 20ppi (filled squares) and 40ppi (open squares). Each value is the mean of three replicates +/- s.e..

Section 3.3 COMPARISON OF THE TROPANE ALKALOID YIELD FROM IMMOBILIZED CELLS OF *ATROPA BELLADONNA* AND *HYOSCYAMUS MUTICUS*

The aims of this experiment were to find out if immobilized cells of *A. belladonna* and *H. muticus* produced tropane alkaloids, and to select the species which accumulated the highest levels for use in subsequent investigations.

The culture medium used was MS (Section 2.2.1.i)), rather than SH medium, because it had previously been used to grow *A. belladonna* cultures which produced alkaloids (e.g. Lindsey & Yeoman 1983a, Eapen *et al* 1978a); and, since it is a more widely used medium than SH, comparison with other work on secondary product formation *in vitro* would be facilitated. The medium was supplemented with the same growth substances as SH medium; $2mgl^{-1}$ CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K (MS-CDK).

The suspension cultures used as sources of inoculum were derived from callus which had been grown on MS-CDK medium for four culture periods, and the comparison was made between cells of the two species at the end of a four week culture period during which immobilization had taken place. The cells were cultured for 26 days rather than 14-21 days as in Section 3.2, in order to allow the cells to reach stationary phase, because, generally, secondary products are accumulated when growth is slow, or has ceased.

In order to assess the effect of immobilization on alkaloid formation, the suspended and immobilized cells from each culture flask were analysed separately. The medium was also extracted to determine if any alkaloid was released.

The newly initiated *A. belladonna* and *H. muticus* suspension cultures were grown for 21 and 15 days, respectively, before use. Ten suspension cultures of each species were used to inoculate ten 250 ml conical flasks, containing 50 ml of MS-CDK medium. Ten ml of each suspension culture was transferred using sterile 25 ml Universal tubes (Sterilin). The mean inoculum size for each species is shown in Table 3.3.1. Ten foam blocks were added to each of the flasks to immobilize the cells as described in Section 2.2.4, before they were incubated under the conditions described in Section 2.2.3.1.

After four weeks, the fresh and dry weights of the immobilized and suspended cells were determined, and used to calculate the relative growth index

(I_{RG}) of the cultures (Section 2.3.2), see Table 3.3.1. Alkaloid extracts were made of the suspended and immobilized cells from each flask (Section 2.4.1.1), and from the pooled spent medium from each species (Section 2.4.1.2). These extracts were dissolved in 1 ml of the mobile phase (Section 2.4.3.2) and analysed by HPLC as described in Section 2.4.3.3. Only atropine was found in the extracts, the amounts being shown in Table 3.3.1.

Greater amounts of atropine were accumulated by cultures of *H. muticus* than by those of *A. belladonna*, and therefore this species was used for the following investigation of tropane alkaloid production.

Atropine was found only in the extracts from immobilized cells, and in order to test the hypothesis that immobilization of the cells promoted atropine formation, a suspension culture control was included in the next experiment.

The appearance of the *A. belladonna* cultures in this experiment did not change from that of the stock cultures, but the *H. muticus* cells changed in colour, from bright green to a dark brownish green, 2-3 days after inoculation. Furthermore, when the foam blocks were cut, it was seen that the cells of both species were concentrated in the outer 2-3 mm of the blocks, those in the centre having died or failed to grow. The presence of a large number of foam blocks in the culture flask may have caused this, possibly by affecting the agitation and thus the aeration of the medium. Therefore in the following experiments, five rather than ten foam blocks were used to immobilize cells.

Having selected the highest alkaloid producing species, *H. muticus*, the pattern of alkaloid production and factors controlling it were examined. The first stage of this investigation, described in the following section, was to characterize the production of tropane alkaloids in immobilized cells.

	A. belladonna		*H. muticus*		
GROWTH					
Inoculum f. wt. (g)	2.047	+/- 0.33	2.215	+/- 0.20	
Inoculum dry wt. (mg)	114	+/- 17	100	+/- 8	
I_{RG} (f.wt.)	7.138	+/- 0.50	7.106	+/- 0.16	
I_{RG} (dry wt.)	4.760	+/- 0.16	5.463	+/- 0.22	*
ATROPINE CONTENT ($\mu g g^{-1}$ f.wt.)					
Immobilized Cells	0.668	+/- 0.67	3.520	+/- 0.76	*
Suspended Cells		ND		ND	
Culture Medium		ND		ND	

Table 3.3.1

A comparison of the growth and atropine accumulation in cultured cells of *A. belladonna* and *H. muticus*. Each value is the mean +/- the s.e..

* denotes that there is a difference between the means at the 2 % level of significance.

ND = not detectable.

Section 3.4 AN INVESTIGATION OF THE GROWTH AND ALKALOID CONTENT OF IMMOBILIZED CELLS OF *HYOSCYAMUS MUTICUS* DURING TWO SUCCESSIVE CULTURE PERIODS

In the last experiment (Section 3.3), immobilized cells of *H. muticus* grown in MS-CDK medium accumulated atropine. The principle aim of the experiment described here was to examine the tropane alkaloid content of cells of *H. muticus* during the culture period in which immobilization took place, and after the immobilized cells were transferred to fresh medium. In addition a comparison was made between cultures in which immobilization was taking place, and suspension cultures.

The sources of inoculum for this experiment were ten suspension cultures which had been initiated (Section 2.2.3.3) 24 days previously in MS-CDK medium from callus which had been maintained on MS-CDK. A perforated spoon spatula was used to transfer cells (mean f. wt. 1.101 +/- s.e. 0.079 g) from these suspension cultures to 50 ml of MS-CDK in sixty 250 ml flasks. Five polyurethane foam blocks were added to 40 of these flasks (Section 2.2.4) before they were sealed with aluminium foil and incubated on an orbital shaker as described in Section 2.2.3.1.

On days 4, 8, 12, 16, and, 21, four each of the cultures containing foam blocks and the control suspension cultures were taken from the shaker, and the f. wt. of the suspended and immobilized cells was determined (Section 2.3.1.1). All the cells from each group of four flasks were then pooled together and extracted for analysis of their alkaloid content (Section 2.4.1.1). On day 21 the immobilized cells in the remaining 20 flasks were transferred to fresh MS-CDK medium. These cultures were then incubated on the rotary shaker for up to three weeks, four flasks being harvested on days 24, 28, 32, 36, and 41. The f. wt. of the suspended and immobilized cells was measured and alkaloid extracts made as before, but in addition, the viability (K_R) of the immobilized cells was monitored from days 21-41 (Section 2.3.2.1). Alkaloid extracts were also made from the pooled spent medium from days 21 and 41 (Section 2.4.1.2).

All the tissue and media extracts were analysed by HPLC as described in Section 2.4.3; detectable amounts of tropane alkaloids were not found in any of them.

The results of the growth and viability measurements are shown in Fig.

3.4.1. The pattern of growth is similar to that seen previously in Section 3.2.3, but with a major difference; the culture f. wt. in this experiment did not increase until after day 4, whereas in the experiment described in Section 3.2.3, it had already begun to increase by day 3.

There was no difference between the growth of cultures growing in the presence of foam blocks, and the control suspension cultures until day 21, when the latter had a greater f. wt.. Again there is a difference from the results of the experiment in Section 3.2.3, where f. wt. had apparently ceased to increase by day 14, here it was still increasing on day 21.

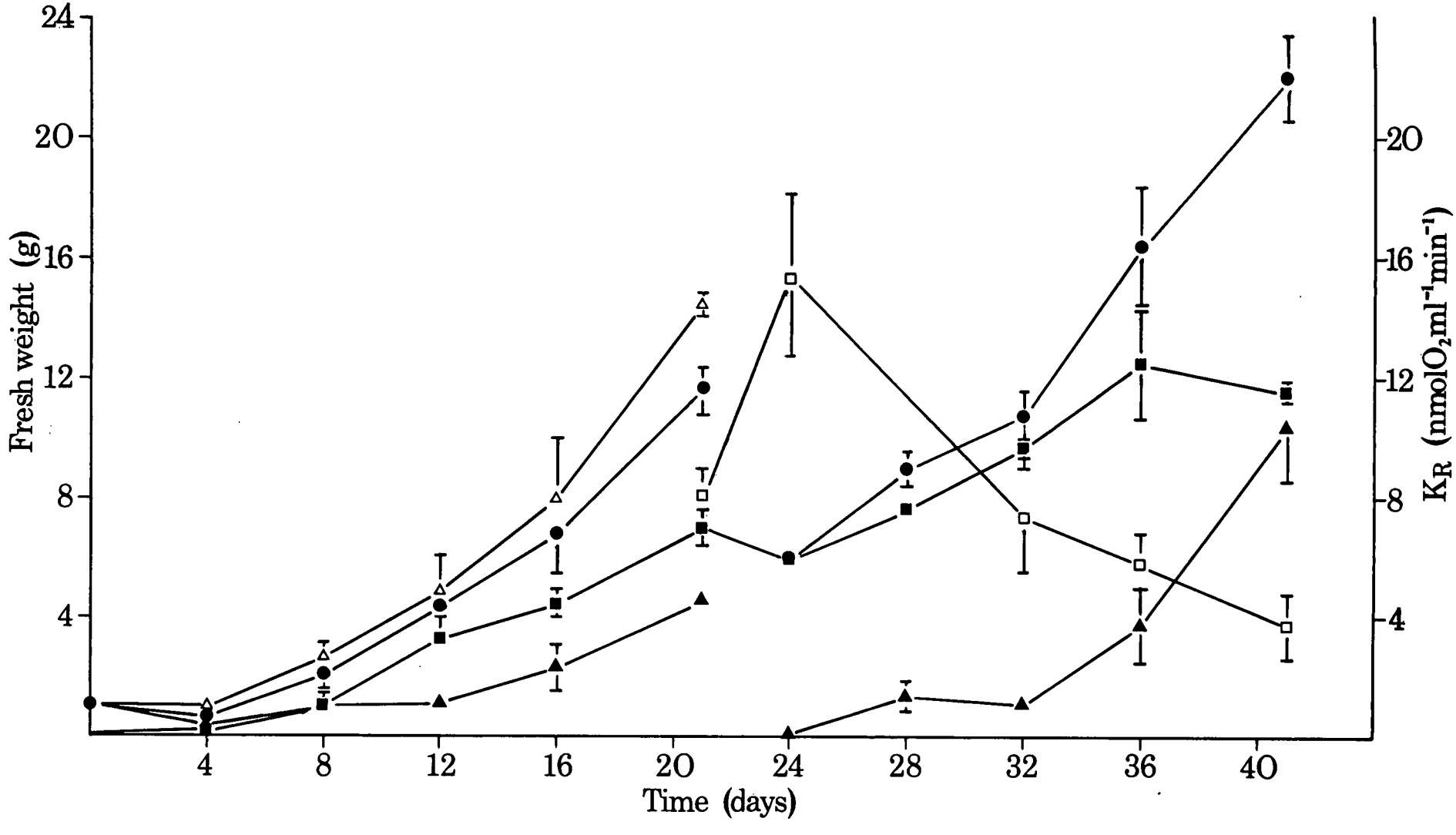
After subculture on day 21, the suspended cell population must have originated from cells lost from the surfaces of the foam blocks. The f. wt. of this population remained low for eight days, then began to rise at approximately the same time that the immobilized f. wt. became stable, day 32.

The relative growth index was smaller in this culture period; 2.14 cf. 9.63 for the first passage of immobilized cultures, and 12.21 for the control suspension cultures. This was probably due to the high inoculum weight of the second culture period and possibly also to the slower growth of immobilized cells.

Since the cells in this experiment did not produce detectable levels of tropane alkaloids, the principle aim of the next series of experiments was to find out if the cells were still capable of producing alkaloids. They could have permanently lost all or part of the tropane alkaloid biosynthetic pathway, or have simply stopped expressing it. Therefore, in the experiments described in the next section, alkaloid precursors were added to the medium, and their effect on alkaloid production was determined.

Fig. 3.4.1

The graph shows the f. wt. of immobilized (filled squares), suspended (filled triangles), and total (filled circles) cells in cultures of *H. muticus* containing five foam blocks, during a 41 day period, with transfer of the foam blocks (containing cells) to fresh medium on day 21. The change in the f. wt. of cells in control suspension cultures of *H. muticus* during the first 21 days (open triangles), and the viability (K_R) of the immobilized cells after subculture (open squares), are also shown. Each value indicates the mean of four replicates +/- s.e..


Fresh weight (g)
K_R (nmolO_2ml^{-1}min^{-1})
Time (days)
4
8
12
16
20
24
28
32
36
40

Section 3.5 THE EFFECT OF ADDED TROPANE ALKALOID PRECURSORS ON THE GROWTH AND ALKALOID ACCUMULATION OF CELLS OF *HYOSCYAMUS MUTICUS*

Tropane alkaloids were not detected in the last experiment (Section 3.4). Therefore, in order to find out if the immobilized cells of *H. muticus* were still able to synthesise tropane alkaloids, precursors were added to the culture medium. In addition, it was possible that by feeding precursors, some indication of the limiting steps, or at least the limiting branch of the pathway would be identified.

The tropane alkaloid biosynthetic pathway is described and illustrated in Chapter One, where it can be seen that the following substances are precursors of l-hyoscyamine. L-ornithine (L-orn) is the amino acid precursor of tropine, and L-phenylalanine (L-phe) that of tropic acid. Na-phenylpyruvate (Na-ppyr) is possibly an intermediate between L-phe and tropic acid. Hyoscyamine is formed by esterification of tropine and tropic acid.

These precursors had been supplied previously to cell cultures of *Datura innoxia* (Hiraoka *et al* 1973) and *Scopolia parviflora* (Tabata *et al* 1972), with varying results. These authors also found that growth was inhibited, to varying extents, by these substances. In order to detect and assess any inhibition of the growth of cells of *H. muticus*, the precursors were first added to the medium of suspension cultures (Section 3.5.1), before their effect on alkaloid production by immobilized cells was determined.(Section 3.5.2).

Section 3.5.1 THE EFFECT OF ADDED TROPANE ALKALOID PRECURSORS ON THE GROWTH OF SUSPENDED CELLS OF *HYOSCYAMUS MUTICUS*

The aim of this experiment was to determine the effect of various tropane alkaloid precursors on the growth (I_{RG}) of suspended cells of *H. muticus*.

Their effect on suspended rather than immobilized cells was determined in order to find an appropriate precursor concentration to use in a subsequent large scale experiment with immobilized cells; and, since suspended cells are in more direct contact with the medium, any effect of the precursors on growth would be

greater and therefore easier to detect and measure.

It is important to measure the effect of the precursors on growth because any change in alkaloid production brought about by their addition to the medium may be caused directly, by supplying substrates for the enzymes of the pathway, or indirectly, by affecting the growth and/or the general metabolic state of the cells.

The precursors were added at two concentrations, 10^{-3} and 10^{-4} M, in order to give an indication of the best concentration for use in later experiments with immobilized cells. The criteria for selection were; i) a relatively high precursor concentration, as it was expected that any affect on alkaloid yield would be proportional to precursor concentration; and ii) a concentration which would not substantially inhibit growth, or kill the cells.

Stock solutions (1 $mgml^{-1}$) of the precursors; L-phe, Na-ppyr, L-orn, DL-tropic acid, and tropine (Sigma), were made up in 50 ml of distilled water. Na-ppyr and DL-tropic acid were first dissolved in c. 5 ml of M KOH. The appropriate amount of each stock solution, to give a final concentration of 10^{-4} or 10^{-3} M, was added to the distilled water used to prepare MS-CDK medium as described in Section 2.2.1.ii). MS-CDK was also made up without precursors as a control medium. Since it was feared that the precursors would be degraded during sterilization by heat (Section 2.2.2.1.i)), all the media were sterilized by filtration (Section 2.2.2.1.ii)).

Aliquots (20 ml) of the medium for each treatment were then placed in four 100 ml conical flasks to each of which was added a known wet wt. (mean 1.039 +/- s.e. 0.032 g) of cells from 21 day old suspension cultures grown in MS-CDK medium. The flasks were then incubated on a rotary shaker as described in Section 2.2.3.1. After 21 days the f. wt. of the cells in each flask was determined and the I_{RG} calculated (Section 2.3.2), the results being shown in Table 3.5.1.

None of the treatments caused differences in the colouration or the texture of the cell cultures. No value for the standard error is given for the effect of treatment with 10^{-3} M tropine because three of the four replicates were lost due to microbial contamination. Growth (I_{RG}) was not significantly affected by any of the precursors at a concentration of 10^{-4} M. At 10^{-3} M L-phe and DL-tropic acid did inhibit growth, the mean I_{RG} being reduced by 41 % and 48 %, respectively, compared with the control. However this inhibition of growth was

not substantial, and therefore 10^{-3} M was the concentration at which precursors were supplied to immobilized cells in the experiments described in the following section.

Treatment	Relative Growth Index (mean +/- s.e.)	Percentage Difference from the Control
Control	4.608+/- 0.494	0
L-phe 10^{-4} M	6.199+/- 0.762	34
L-phe 10^{-3} M	2.679+/- 0.363 *	-41
Na-ppyr 10^{-4} M	5.793+/- 0.614	26
Na-ppyr 10^{-3} M	3.306+/- 0.799	-28
L-orn 10^{-4}	3.002+/- 0.110	-35
L-orn 10^{-3} M	3.672+/- 0.235	-20
DL-tropic acid 10^{-4} M	4.203+/- 0.593	-9
DL-tropic acid 10^{-3} M	2.393+/- 0.111 **	-48
tropine 10^{-4} M	4.007+/- 0.787	13
tropine 10^{-3} M	8.475	84

Table 3.5.1
The effect of added tropane alkaloid precursors on the relative growth index of suspension cultures of *H. muticus*.

* denotes mean values significantly different from the control at the 5% level, ** those different at the 1% level.

Section 3.5.2 EXAMINATION OF THE EFFECT OF ADDED PRECURSORS ON TROPANE ALKALOID PRODUCTION BY IMMOBILIZED CELLS OF *HYOSCYAMUS MUTICUS*

In the experiment described in the previous section the effect of tropane alkaloid precursors added to the culture medium (MS-CDK) on the growth of suspended cells was determined, and 10^{-3} M found to be a suitable concentration with which to examine their effect on alkaloid production by immobilized cells.

Cells which were already immobilized were transferred to fresh MS-CDK medium (the control treatment), or to MS-CDK containing one of the precursors; L-phe, L-orn, Na-ppyr, DL-tropic acid, or tropine, at 10^{-3} M. At the end of the culture period their growth and alkaloid content were measured and compared.

The I_{RG} was not used as a measure of growth here, because the weight of the immobilized cells was not determined at the beginning of the culture period. Instead, since all the cultures in which immobilization had taken place had a similar inoculum size and were subsequently grown under the same conditions, except for the treatment media, the final f. wt. was used to compare the effect of the precursors on the growth of immobilized cells.

Since tropane alkaloids were not detected in the experiment described in Section 3.4, the alkaloid content of the cells was again expected to be low, if it was detectable. Therefore all the cells from each treatment were pooled together for alkaloid extraction, to facilitate analysis.

Due to microbial contamination, all the cultures fed with DL-tropic acid were lost. Therefore a second experiment was performed to determine the effect of DL-tropic acid. In addition to the control of MS-CDK medium, two other treatments were included in this experiment; 10^{-3} M tropine, and 10^{-3} M tropine + 10^{-3} M DL-tropic acid.

Cells for both experiments were first immobilized in five foam blocks in 50 ml of MS-CDK medium in 250 ml flasks, as described in Section 2.2.4. The cells, mean wet wt. 1.194 +/- s.e. 0.04 g in the first experiment, 1.210 +/- s.e. 0.060 g in the second, were taken from suspension cultures grown in MS-CDK medium, which had been initiated 14 days previously from callus maintained on MS-CDK medium. Cells were immobilized for 14 days, then the five foam blocks from each flask (containing cells) were transferred to a 250 ml flask containing 50 ml

of one of the treatment media, there being five flasks per treatment. The treatment media had been prepared and sterilized as described in Section 3.5.1. The flasks were then incubated on a rotary shaker under the conditions described in Section 2.2.3.1.

After 24 days the cultures were harvested, and the f. wt. of the cells in each flask was determined (Section 2.3.1.1) before all the cultured cells from each treatment were pooled together for alkaloid extraction (Section 2.4.1.1). The alkaloid extracts were stored in 5 ml of MeOH. Prior to HPLC analysis (Section 2.4.3.3), the MeOH was evaporated from 4 ml of each extract and the residue taken up in 1 ml of the HPLC mobile phase in the manner described in Section 2.4.3.2. Only atropine was found in the extracts.

The results of the two experiments are shown in Tables 3.5.2 and 3.5.3. They show that the growth (final f. wt.) and the atropine content ($\mu g g^{-1}$ f. wt.) of the control cells from both experiments were similar. Contrary to the results with suspended cells, described in Section 3.5.1, L-phe and DL-tropic acid did not inhibit the growth of cells. Tropine did reduce the growth, but only in the second experiment, when there was a 24% reduction of the mean f. wt. compared with the control (Table 3.5.3). This variable effect of tropine on growth may be connected with its different effect on alkaloid accumulation in the two experiments. In the first experiment (Table 3.5.2) it caused a slight decrease in the atropine content of the cell compared with the control, but in the second experiment (Table 3.5.3) it increased the yield per g f. wt. by approximately 40%.

L-phe and DL-tropic acid caused the greatest increases in alkaloid levels, 115% and 57%, respectively. These values cannot be used to assess the relative efficiency of these precursors as promoters of alkaloid accumulation, as they are derived from different experiments, experiments in which tropine had different effects.

L-orn reduced the atropine content by approximately 80%, while Na-ppyr reduced it to an undetectable level.

When tropine and DL-tropic acid were fed together, they had an additive effect on the atropine concentration of the cells.

Overall the results show that the tropane alkaloid pathway was still active in the cells, and that the amount of alkaloid accumulated can be increased by supplying certain precursors to the cells. However, more experiments are required to define the effects of the precursors, and to indentify the limiting steps of the pathway. These are discussed in relation to the results already obtained in Chapter Four.

Having found that the cells were able to synthesise atropine, an attempt was made to increase the amount produced by exposing the cells to nutrient stress. This was expected to suppress the cell growth and limit primary metabolism, particularly protein synthesis; and thus to promote secondary metabolism, which is generally considered to be inversely related to these processes.

Treatment	Final f.wt. (mean +/- s.e.)	Alkaloid Content of Tissue ($\mu g g^{-1}$ f.wt.)	Percentage of Control
Control	18.447 +/- 2.892	0.894	100
L-phe	16.290 +/- 2.176	1.923	215
Na-ppyr	13.775 +/- 3.015	ND	ND
L-orn	18.363 +/- 3.790	0.194	22
DL-tropic acid	-	-	-
tropine	16.295 +/- 2.787	0.755	84

Table 3.5.2
The effect of tropane alkaloid precursors at 10^{-3} M on the final culture f. wt. and the atropine content of immobilized cells of *H. muticus*.

Treatment	Final f.wt. (mean +/- s.e.)	Alkaloid Content of Tissue ($\mu g g^{-1}$ f.wt.)	Percentage of Control
Control	22.683 +/- 1.302	0.908	100
DL-tropic acid	19.035 +/- 1.421	1.424	157
Tropine	17.135 +/- 1.356 *	1.285	142
DL-tropic acid and Tropine	18.617 +/- 1.322	1.880	220

Table 3.5.3
The effect of the immediate precursors of atropine on the final f. wt. and the atropine content of immobilized cells of *H. muticus*.

* denotes a mean value different from the control at the 2 % level of significance.

Section 3.6 THE EFFECT OF NUTRIENT STRESS ON CELLS OF *HYOSCYAMUS MUTICUS*

In the last series of experiments, immobilized cells in medium without added precursors produced atropine, showing that the tropane alkaloid pathway was active. The experiments described in this section were designed to discover if the levels of alkaloids accumulated by these cells could be increased by incubating them in media which were devoid of certain macronutrients, inorganic nitrogen (i-N), and inorganic phosphate (i-P).

Generally, high levels of i-N and i-P support rapid growth, while their depletion or deficiency is associated with growth limitation and increased secondary metabolite production (Mantell & Smith 1983). It is possible that this is mediated by restricting growth or by directing amino acids from protein synthesis to secondary metabolic pathways.

Previously, limitation of i-P (Mantell & Smith 1983, Knobloch *et al* 1981), i-N (Westcott & Henshaw 1976, Amorim *et al* 1977, Yeoman *et al* 1980), and i-N + i-P (Hall & Yeoman 1986) increased the secondary metabolite yield from cultured cells.

The strategy employed here was to examine the effect on suspended cells of leaving out i-P and/or i-N from the medium (Section 3.6.1) to assess the effect on growth. Then, using media which inhibited the growth of, but did not kill, suspended cells, an experiment was performed to determine the effect of nutrient stress on immobilized cells (Section 3.6.2).

Section 3.6.1 THE EFFECT OF i-P AND i-N LIMITATION ON THE GROWTH OF SUSPENDED CELLS OF *HYOSCYAMUS MUTICUS*

The response of cells of *H. muticus* to media without i-N and/or i-P was examined in the experiment described in this section. The aims were to discover if cell viability was substantially reduced in these media, and to determine their effect on growth. Suspended cells were used, as, being in more direct contact with the medium than immobilized cells, the effects of the media on growth and viability would be more pronounced and easier to detect and evaluate.

Three nutrient limited media were tried: MS medium lacking in i-N, MSN; MS lacking in i-P, MSP; MS and lacking in both i-N and i-P, MSNP; as described in Section 2.2.1.iii). The control medium was MS-CDK (Section 2.2.1.ii)). Five 20 ml aliquots of each medium were placed in 100 ml conical flasks which were then sealed with aluminium foil and sterilized by heat (Section 2.2.2.1.i)).

A known wet wt. of cells (mean 1.165 +/- s.e. 0.056 g) was added to each flask, from ten suspension cultures grown in MS-CDK, which had been initiated 21 days previously from callus maintained in MS-CDK. After being incubated on an orbital shaker (Section 2.2.3.1) for two weeks, the f. wt. of the cells was determined (Section 2.3.1.1) and used to calculate the relative growth index, I_{RG} (Section 2.3.1.4), the results are shown in Table 3.6.1. On preparation, the pH of MSNP had been difficult to adjust, due to the lack of buffering capacity normally supplied by i-P. In order to ensure that any effect on growth of the cells was due to nutrient limitation and not to a change in pH that was deleterious to the cells, the medium pH was determined at the time of harvest, and is included in Table 3.6.1. Visible differences can be seen in the appearance of the cells between the treatments, these are shown in Fig 3.6.1.

Any effect of the media on growth cannot have been due to an alteration in pH, as this was similar in all the treatments. MSN medium caused a reduction in growth by a factor of 10, and the cultures were composed mainly of very compact aggregates, although the colouration was similar to the control cells. The negative values of I_{RG} for cells treated with MSP and MSNP do not necessarily mean that there was a loss of f. wt. during the culture period, because I_{RG} was calculated using an initial wet wt. and a final f. wt. (Section 2.3.1.4). However growth in these treatments was probably inhibited more or less completely. Although the mean I_{RG} was smaller in MSNP, than MSP, there is no significant difference between the values. The cells from both these treatments had a distinctly brownish colouration, particularly those grown in MSP, but on microscopic examination there were few dead cells, which indicates the accumulation of polyphenols in these cells.

Thus complete withdrawal of i-N and/or i-P from the medium caused a marked reduction in growth, but did not appear to reduce viability. Therefore these media were used to examine the effect of nutrient stress on immobilized cells (Section 3.6.2). In addition the respiration rate (K_R) of the cells was

determined in order to assess the effect of the treatments on viability.

Treatment Medium	Relative Growth Index (mean +/- s.e.)	Medium pH
MS-CDK	3.188+/- 0.422	5.3
MSN	0.305+/- 0.447 ***	5.0
MSP	-0.307+/- 0.087 ***	5.1
MSNP	-0.143+/- 0.086 ***	5.1

Table 3.6.1
The effect of MSN, MSP, and MSNP media, compared with MS-CDK medium as control, on the Relative Growth Index and the final medium pH of suspension cultures of *H. muticus*.

*** denotes mean values significatly different from the control at the 0.1% level.

Fig. 3.6.1

The appearance of suspension cultured cells of *H. muticus* grown in MS, MSN, MSP, and MSNP media for two weeks.

Section 3.6.2 THE EFFECT OF MEDIA LACKING IN NITROGEN AND/OR PHOSPHATE ON IMMOBILIZED CELLS OF *HYOSCYAMUS MUTICUS*

Having found that MSN, MSP, and MSNP media inhibited the growth of suspension cultures, without killing the cells, their effect on the alkaloid content of immobilized cultures was investigated.

Five 14 day old suspension cultures, grown in MS-CDK medium, which had been initiated from callus maintained on the same medium were used as the inoculum for 16 immobilized cultures. The cells were immobilized as described in Section 2.2.4, using five foam blocks per flask, the mean wet wt. of the inoculum being 1.278 +/- s.e. 0.056 g. Immobilization was allowed to take place for 21 days, then the immobilized cells from each flask were transferred to 50 ml of one of the treatment media in a 250 ml flask, there being four flasks per treatment. The nutrient limiting media; MSN, MSP, and MSNP had been prepared as described in section 2.2.1.iii), MS-CDK, the control medium, as described in Section 2.2.1.ii). The flasks were then placed on a rotary shaker and cultured under the conditions detailed in Section 2.2.3.1 for 21 days.

Immediately after the removal of each flask from the shaker, one foam block was taken, and its wet wt. was determined before the specific respiration rate (K_R) of the cells in it was measured (Section 2.3.2.1). The f. wt. of the suspended and immobilized cells from each flask was determined (Section 2.3.1.1.) before all the cells from each treatment were pooled together and extracted (Section 2.4.1.1) for analysis.

Since the inoculum size was similar in all the cultures at the onset of immobilization and they were all grown under the same conditions, the final f. wt. of the suspended, immobilized, and total cells shows the effect of nutrient stress on growth. The results of the growth and viability measurements are shown in Table 3.6.2.

All the nutrient limited media inhibited the growth of the immobilized cells. But contrary to the results of the last experiment, there was no significant difference between the f. wt. of cultures grown in MSN medium and those grown in MSP and MSNP media. There was less growth of suspended cells in cultures grown in MSN medium than in all the other treatments, which reflects the very compact, infriable nature of cultures in this medium.

The K_R of the cells was not significantly reduced by any of the treatments compared with the control, but those treated with MSP medium had a higher K_R than the other cultures. This could be related to changes in the metabolism of these cells, which again had a distinct brown colouration, indicating an increased production of polyphenols (Fig 3.6.1).

None of the extracts from the cells contained tropane alkaloids. This shows that nutrient stress does not induce alkaloid production, but more experiments must be performed to find out if it can increase the amount of alkaloid produced by cells which already accumulate detectable amounts. However at this point attention was centred not on culture growth but on another factor which may control alkaloid production, differentiation. Stock cultures of *H. muticus* were induced to differentiate by manipulation of the growth substances added to the medium.

Treatment	Suspended Cells (g f. wt.)	Immobilized Cells (g f. wt.)	Total Cells (g f. wt.)	I_{RG} ($nmolO_2$ $ml^{-1}min^{-1}g^{-1}$)
Control	5.209	13.187	18.396	2.458
MSN	0.128 **	8.493 **	8.621 **	3.366
MSP	0.746	5.764 ***	6.510 **	5.970 *
MSNP	1.958	5.093 ***	7.051 ***	2.238

Table 3.6.2
The effect of nutrient limiting media on the growth and viability (K_R) of immobilized cultures of *H.muticus*, compared with control cultures in MS-CDK medium. Each value is the mean of five replicates.

*, **, and *** indicate means which are significantly different from the control values at the levels of 5%, 1%, and 0.1%, respectively.

Section 3.7 AN EXAMINATION OF THE RELATIONSHIP BETWEEN DIFFERENTIATION AND TROPANE ALKALOID ACCUMULATION IN IMMOBILIZED CELL CULTURES

The formation of certain secondary products *in vitro* relies not just upon slow growth, but is linked to differentiation of the cell cultures (see Chapter One). Such a relationship has been reported between alkaloid production and differentiation in cultures of *A. belladonna*. Tropane alkaloid production was thought to be linked specifically to root formation because they were only detected in roots or root callus (West & Mika 1957); in excised roots or roots regenerated from callus (RajBhandary *et al* 1969); or in suspension cultures which formed roots (Thomas & Street 1970). Furthermore, although there was no difference in the alkaloid content of the unorganized or the highly differentiated cultures of Eapen *et al* (1978a), the structures formed in these cultures were always shoot buds, not roots. However, Lindsey & Yeoman (1983a) reported a greater alkaloid content in a highly embryogenic culture than in undifferentiated suspension cultures, which suggested that embryogenesis in addition to root formation may be linked to alkaloid production.

There are no reports of any similar studies with *H. muticus*, therefore the aims of the experiments described in this section were to induce differentiation in immobilized cells of *H. muticus* and to examine the effect of this on their alkaloid production.

First the effect upon callus and suspension cultures of changes in the amounts of growth substances added to the medium was assessed in terms of the resulting differentiation (Section 3.7.1). Then immobilized cells were exposed to changes in both the amount and type of growth substances added to a number of different basal media, and the morphogenesis and alkaloid production in these cultures was examined (Section 3.7.2).

Section 3.7.1 THE INDUCTION OF STRUCTURAL DIFFERENTIATION IN CALLUS AND SUSPENSION CULTURES OF *HYOSCYAMUS MUTICUS*.

The basic strategy for the induction of structural differentiation in plant cell cultures, is to transfer them from a primary medium, which contains an auxin and a cytokinin, to a secondary (induction) medium in which the auxin has been withdrawn or reduced in concentration, and the cytokinin concentration has been maintained or increased (Thomas & Street 1970, Gosch *et al* 1975).

Cell cultures of *A. belladonna* may react in various ways to changes in the amounts of growth substances added to the medium: forming roots (RajBhandary *et al* 1969; Thomas & Street 1970), shoot buds (Eapen *et al* 1978a), and embryos (Gosch *et al* 1975). Lorz *et al* (1979) induced organogenesis in cultures of *H. muticus* using a range of treatments, but reported only shoot formation. Thomas & Street (1970), and Eapen *et al* (1978a) found that there was a decrease in, or loss of, the ability to differentiate with the increasing number of passages which the cultures had been through prior to exposure to the induction medium.

In the experiment described here, callus and suspension cultures of *H. muticus* were subjected to media devoid of auxin and containing various amounts of cytokinin; the aim being to determine if the cultures of *H. muticus* which had been initiated over one year previously, were still capable of structural differentiation, and, if so, to find out what structures were formed in callus and suspension cultures by simple manipulation of the growth substances added to the medium.

Callus and suspension cultures (five replicates of each) were grown in MS-CDK medium (Section 2.2.1.i)) for three weeks, then 1-2 g f. wt. of cells were transferred from each of the replicates into the four secondary media. These were MS-CDK, the control, and three secondary induction media, MS medium lacking in auxin (CPA and 2,4-D) but containing 0.1, 0.2, and 1.0 mgl^{-1} K. Observations were made four weeks later with the aid of a binocular microscope.

Callus Cultures

There was no structural differentiation in the control cultures, but in those grown on induction media, shoots and, very hairy, root apices could be seen on the surface of the callus, and more were seen within the callus when it was disrupted. In the callus grown on MS with 0.1 mgl^{-1} K, there were many root and shoot apices, root apices being slightly more common. At twice this concentration of

K, there was less organogenesis, and shoot apices were more frequent. In the presence of 1.0 mgl^{-1} K there was more variation between replicates in the amount of organogenesis observed, but it was generally less than in the other two treatments. Again, shoots predominated over roots, but there were also many undeveloped apices, and abnormal structures ,e.g. thickened roots.

Suspension Cultures

Again there was no structural differentiation in the control cultures. In the induction media, all the replicates formed only roots. These roots were apparently hairless, and were often longer (more than 2 mm in length) than those in the callus cultures. An inverse relationship may exist between the number of roots formed and their length, and the K concentration, since many roots in 0.1 mgl^{-1} K were over 5 mm in length, while in 1.0 mgl^{-1} K they were approximately 2 mm in length. In the latter medium, some roots had again become thickened, and there were a number of compact, bright green aggregates.

Therefore the results show that the *H. muticus* cultures have not lost the ability to differentiate structurally, despite their age; and that roots and shoots were formed in callus, but only roots in suspension cultures. Also it is possible that the K concentration may affect root development.

In the experiment with immobilized cells described in the next section, the same treatment of auxin withdrawal and changing cytokinin concentration was applied, but was coupled with an examination of the effects of the basal medium, and of the type of auxin added to the primary medium, to find out if they could alter the amount of morphogenesis that occurred and the type of structures that were formed. The tropane alkaloid content of these cultures was also analysed, to determine if there is any correlation between structural differentation and alkaloid production in immobilized cultures of *H. muticus*.

Section 3.7.2 AN EXAMINATION OF THE RELATIONSHIP BETWEEN STRUCTURAL DIFFERENTIATION AND ALKALOID PRODUCTION IN IMMOBILIZED CULTURES OF *HYOSCYAMUS MUTICUS*

In this experiment alkaloid content and the organogenesis induced in immobilized cultures by a variety of treatments were examined to determine if there was a relationship between them.

As before, secondary induction media devoid of auxin, and containing different levels of cytokinin were used; but the effects of the type of auxin contained in the primary medium, and of different basal media were also examined. NAA was used as an alternative to the auxins CPA and 2,4-D in the primary medium, as this was the auxin used by RajBhandary *et al* (1969), Thomas & Street (1970, 1972), and Lorz *et al* (1979). The basal media used were MS, SH, and SSM, the medium used by Thomas & Street (1970, 1972). The latter medium (SSM) was included because in this formulation, cultures of *A. belladonna* formed embryos in addition to roots and shoots, and therefore if cultures of *H. muticus* reacted similarly, the effect of embryogenesis cf. organogenesis could be investigated. Thomas & Street (1972) added various amounts of ammonium sulphate to their SSM medium. In this experiment the amount added gave a similar ammonium ion concentration to that in MS medium. Thus there were six primary media; SH, MS, and SSM (whose constituents are given in Table 2.2.1) each supplemented with 2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K (CDK) and with 1.0 mgl^{-1} NAA and 0.1 mgl^{-1} K (NK); and they were termed SH-CDK, SH-NK, MS-CDK, MS-NK, SSM-CDK, and SSM-NK.

In the last experiment, morphogenesis was assessed only in a qualitative manner, but here, as treatments were to be compared, and as a possible relationship with alkaloid production was anticipated, the organs which formed in these immobilized cultures were counted.

Suspension cultures were initiated (Section 2.2.3.3) from stock callus grown in SH medium in all of the primary media and grown for two weeks. Then these suspension cultures were subcultured into fresh medium (ten flasks per medium) and five foam blocks were added to each flask to immobilize the cells (Section 2.2.4). Immobilization took place over three weeks, then the foam blocks, containing cells, were transferred to secondary media. There were three secondary media for each primary medium; the control medium, which contained the same growth substances as the primary medium (four flasks), and two secondary induction media (three flasks of each) which were both lacking in auxin, but contained 0.1 and 1.0 mgl^{-1} K.

The cultures were incubated on a rotary shaker under the conditions described in Section 2.2.3.1, and harvested 21 days later. A qualitative description of the cultures was made, before the number of roots (no shoots were found) produced by the immobilized cells in three foam blocks from each flask

were counted. This figure was used to derive a value for the number of roots per g f. wt. of immobilized cells, after the f. wt. of the suspended and immobilized cells in each flask had been determined (Section 2.3.1.1). All the cells from each secondary medium were then pooled together, and extracted as described in Section 2.4.1.1, the extracts being stored in 5 ml of MeOH. Prior to analysis by HPLC (Section 2.4.3.3), the samples were prepared as described in Section 2.4.3.2, 4 ml of each extract being dried down and taken up in 1 ml of the mobile phase. Only atropine was detected in the extracts. Table 3.7.1 shows the f. wt. of the suspended, immobilized, and total cells in each secondary medium. The number of roots per g immobilized f. wt., together with the atropine content of the cultures (in $\mu g g^{-1}$ f. wt.) are shown in Table 3.7.2.

The cultures previously grown on the primary medium SH-CDK (Fig 3.7.1) were yellow-green in colour and friable. There was a great deal of outgrowth of cells on the foam blocks and a large population of finely suspended cells. After growth in SH-NK (Fig 3.7.2), the colour of the cells was a slightly darker, brownish green, and the suspended cells were more aggregated, but still quite friable. There was a marked difference in appearance between cultures grown in the primary media MS-CDK and MS-NK (Figs 3.7.3 and 3.7.4). Those from MS-CDK had a large population of finely suspended cells, and outgrowth of friable cells on the foam blocks, while cultures grown in MS-NK were bright green, and compact, and there were fewer suspended cells which were highly aggregated. When cell suspension cultures were first initiated in SSM medium, their growth was similar to cells in SH or MS medium, and they were green. However, with successive culture passages in SSM, they grew less, and became cream and/or brown in colour (Figs 3.7.5 and 3.7.6). The effect on cell growth of this medium is illustrated by the f. wt. of cells grown in SSM being lower than in other media (Table 3.7.1).

Cells grown in SSM did not produce detectable amounts of alkaloids, and formed few roots (Table 3.7.2). No roots were formed at all in SH-CDK, MS-CDK or SSM-CDK, but they were formed in all the other secondary media. In cultures from each group of three secondary media, the largest number of roots was formed in the presence of 0.1 mgl^{-1} K. However, there is no clear pattern in the effect of the growth substances incorporated in the primary media. Cells cultured originally in the primary medium MS-NK formed less roots than those from MS-CDK, while those from SH-NK produced more roots than those from the primary medium SH-CDK. Fig 3.7.7 shows one of the best examples of root formation by cells in MS-K, cultured first in the primary medium MS-NK.

Although this experiment was designed to examine the relationship between alkaloid production and root formation, it also provided additional information about the effect of the basal medium and the growth substances added to it. In SSM medium no alkaloids were formed. This is probably because it not only limited growth (Table 3.7.1), but also decreased the viability of the cells. The carry over effect of media is also illustrated. Initially suspension cultures in SSM, derived from stock callus on SH medium, appeared to be as healthy as those in SH or MS medium, but after three passages (when the cells were harvested) the adverse effects of this medium could be seen. No clear difference in the amount of alkaloid producted or the incidence of organogenesis can be seen between cultures grown in SH and MS media.

No firm conclusions can be drawn concerning the effect of growth substances on alkaloid production, since the replicate cultures were pooled together for analysis. However the optimum growth substance supplementation for root formation, i.e. 0.1 mgl^{-1} K in medium without auxin, does not appear to be optimum for alkaloid yield. The highest alkaloid contents were seen in cultures grown in the control media, i.e. in media containing auxin and 0.1 mgl^{-1} K. The effects of increasing the K concentration in the absence of auxin are not consistent between the groups of secondary media, an increase, a decrease, and no change being seen in the alkaloid content of cultures. More experiments are required to determine if the withdrawal of auxin decreases atropine production by cell cultures, and if the concentration of K can affect the yield.

In order to ascertain whether there was any relationship between root formation and alkaloid accumulation, the atropine content per g f. wt. was plotted against the number of roots per g f. wt. (Fig 3.7.8), and the regression line of atropine content on the no. of roots was fitted (Section 2.6.2). There is no correlation between them as the regression coefficient was not significantly different from zero.

However, there was much variation in the number of roots formed among the replicates in each secondary medium, and this could not be directly related to alkaloid production, as all the replicates were pooled together for alkaloid analysis. Therefore in order to be sure that there is no link between differentiation of roots and alkaloid production, further experiments, were required to compare the number of roots and the amount of alkaloid formed within each replicate flask. Furthermore any such relationship would be more easily detected and evaluated if more organogenesis occurred in the cultures, and

if comparisons could be made between cultures grown in one rather than in a number of media. An attempt was therefore made to isolate culture lines with an increased ability to form roots (Section 3.8). Alkaloid accumulation and organogenesis could then be compared between these culture lines.

Primary Medium	Growth Substances in Secondary Medium	Fresh Weight of Cells mean +/- s.e. (g) Suspended	Immobilized	Total
SH-CDK	CDK	8.882 +/- 2.96	11.256 +/- 0.58	20.139 +/- 2.39
SH CDK	K	11.832 +/- 4.00	7.248 +/- 0.71	19.081 +/- 4.68
SH-CDK	10K	11.500 +/- 0.30	12.958 +/- 1.48	24.518 +/- 1.23
SH-NK	NK	11.396 +/- 0.30	7.321 +/- 0.69	18.716 +/- 0.38
SH-NK	K	3.994 +/- 2.71	7.601 +/- 0.32	11.595 +/- 2.66
SH-NK	10K	6.611 +/- 3.15	6.967 +/- 0.73	13.578 +/- 2.52
MS-CDK	CDK	9.057 +/- 1.67	10.323 +/- 1.17	19.380 +/- 1.50
MS-CDK	K	9.066 +/- 1.15	7.321 +/- 0.84	16.387 +/- 1.84
MS-CDK	10K	10.407 +/- 1.54	9.220 +/- 1.07	19.627 +/- 2.54
MS-NK	NK	3.217 +/- 0.82	7.889 +/- 1.75	9.106 +/- 0.52
MS-NK	K	3.370 +/- 0.38	10.082 +/- 2.79	13.458 +/- 2.65
MS-NK	10K	5.327 +/- 1.71	10.958 +/- 1.34	16.285 +/- 2.04
SSM-CDK	CDK	5.791 +/- 0.68	5.178 +/- 0.77	10.969 +/- 1.37
SSM-CDK	K	3.056 +/- 0.05	4.913 +/- 0.01	7.968 +/- 0.07
SSM-CDK	10K	2.472 +/- 0.33	4.841 +/- 0.87	7.313 +/- 1.17
SSM-NK	NK	0.959 +/- 0.13	5.062 +/- 0.49	6.074 +/- 0.64
SSM-NK	K	1.340 +/- 0.65	6.338 +/- 2.09	7.678 +/- 2.74
SSM-NK	10K	0.713 +/- 0.01	5.286 +/- 0.82	5.994 +/- 0.81

Table 3.7.1

The f. wt. of suspended, immobilized, and total cells in cultures of *H. muticus* which were subjected to a variety of treatments. The cells were grown in one of the six primary media; SH, MS and SSM supplemented with one of two growth substance combinations, CDK (2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K) and NK (1.0 mgl^{-1} NAA, and 0.1 mgl^{-1} K). They were then transferred to secondary media containing the same growth substance combination, or with just kinetin at the same concentration (K) or at ten fold the original concentration (10K).

Primary Medium	Growth Substances in Secondary Medium	Mean No. of Roots per g immobilized Fresh Weight	Atropine Content $\mu g g^{-1}$ f. wt.
SH-CDK	CDK	0	1.909
SH-CDK	K	2.4	1.210
SH-CDK	10K	0.008	0.9150
SH-NK	NK	3.7	1.642
SH-NK	K	6.3	0.738
SH-NK	10K	3.9	1.555
MS-CDK	CDK	0	3.691
MS-CDK	K	3.9	1.156
MS-CDK	10K	3.1	1.140
MS-NK	NK	1.0	-
MS-NK	K	1.4	1.773
MS-NK	10K	0.6	0.754
SSM-CDK	CDK	0	ND
SSM-CDK	K	0.1	ND
SSM-CDK	10K	0.3	ND
SSM-NK	CDK	0.4	ND
SSM-NK	K	0.5	ND
SSM-NK	10K	0.1	ND

Table 3.7.2

The number of roots formed in the foam blocks, and the amount of atropine produced, per g f. wt. of cells of *H. muticus*, after a variety of treatments. The cells were immobilized in one of six primary media; SH, MS and SSM, supplemented with one of two growth substance combinations, CDK (2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K) and NK (1.0 mgl^{-1} NAA, and 0.1 mgl^{-1} K). They were then transferred to secondary media containing the same growth substance combination, or just kinetin at the same (K) or at ten fold the original concentration (10K).

Fig. 3.7.1
Cells of *H. muticus* immobilized in SH-CDK medium, then cultured for 21 days in fresh medium.

Fig. 3.7.2
Cells of *H. muticus* immobilized in SH-NK medium, then cultured for 21 days in fresh medium.

Fig. 3.7.3
Cells of *H. muticus* immobilized in MS-CDK medium, then cultured for 21 days in fresh medium.

Fig. 3.7.4
Cells of *H. muticus* immobilized in MS-NK medium, then cultured for 21 days in fresh medium.

Fig. 3.7.5
Cells of *H. muticus* immobilized in SSM-CDK medium, then cultured for 21 days in fresh medium.

Fig. 3.7.6
Cells of *H. muticus* immobilized in SSM-NK medium, then cultured for 21 days in fresh medium.

Fig. 3.7.7
Root formation in a culture of *H. muticus* immobilized in MS-NK medium, then grown for 21 days in MS-K medium.

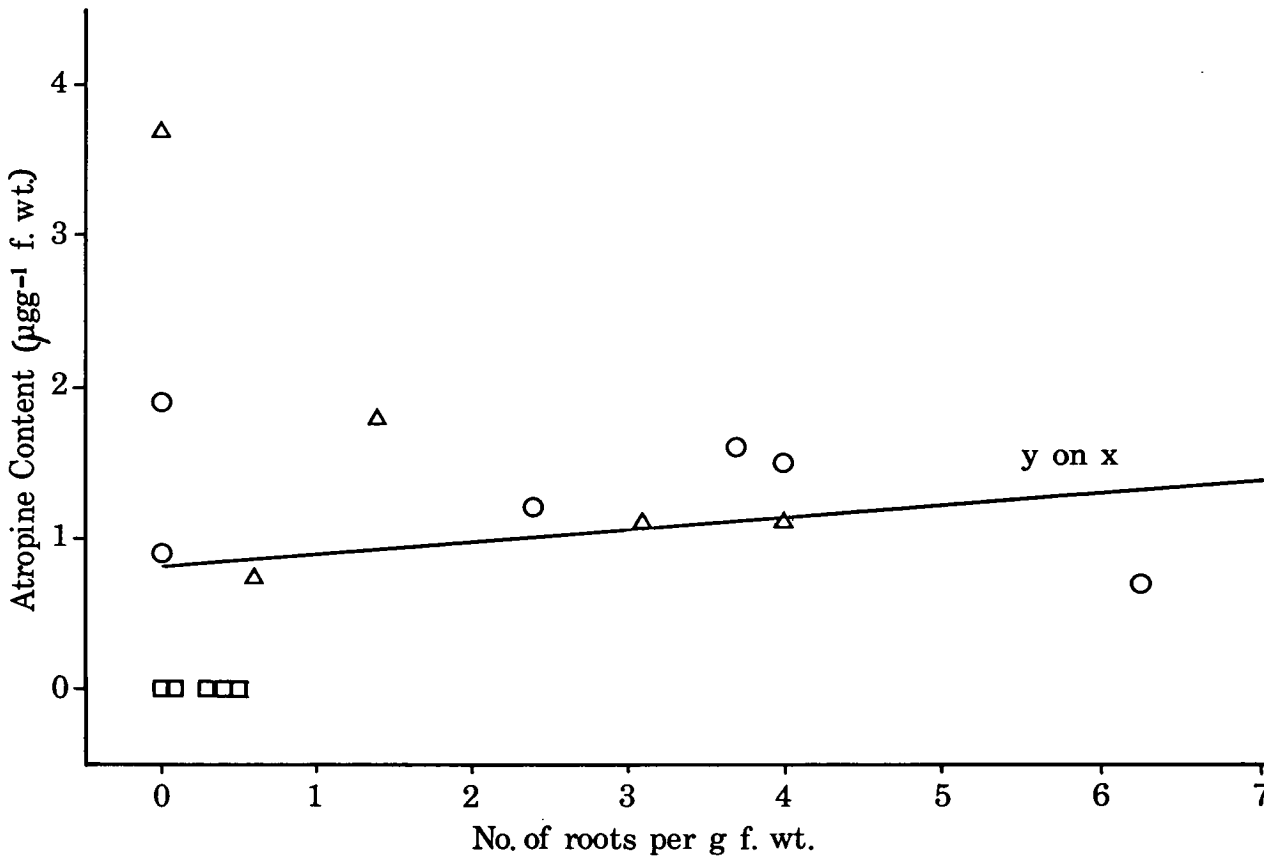


Fig. 3.7.8

A scatter diagram to show the relation between the number of roots per g immobilized f. wt., x, and the atropine content per g f. wt.,y , of cultures of *H. muticus*. Each point shows the mean value of x, and the value of y, for the group of three replicate cultures subjected to each of the treatments described in Section 3.7.2. The different symbols indicate the basal medium in which the cultures were grown; triangles = MS medium, circles = SH medium, and squares = SSM.

The fitted regression line of y on x, whose equation is $y = 0.842 + 0.796x$, is also shown.

Section 3.8 ALKALOID PRODUCTION IN CULTURE LINES OF *HYOSCYAMUS MUTICUS* SELECTED ON THE BASIS OF THEIR ORGANOGENIC ABILITY

In the experiments described in this section, alkaloid production was studied in a number of culture lines with varying abilities to perform organogenesis. Since the amount of organogenesis varied with the culture line rather than with the medium constituents (as in Section 3.7.2), it could be more directly related to alkaloid production.

The origin of the culture lines is described in Section 3.8.1 together with an investigation of their ability to perform organogenesis and produce tropane alkaloids. Following this, a more detailed examination was made of the organogenesis which occurred in these, and in reselected lines in relation to their alkaloid production (Section 3.8.2).

Section 3.8.1 THE SELECTION OF CULTURE LINES AND AN EXAMINATION OF THEIR ORGANOGENIC ABILITY

In the preliminary phase of a planned experiment to further investigate the relationship between structural differentiation and alkaloid production, suspension cultures were initiated in MS medium containing 1.0 mgl^{-1} NAA and 0.1 mgl^{-1} K (MS-NK), from stock callus of *H. muticus* grown in SH medium (supplemented with 2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D and 0.1 mgl^{-1} K). As before (Section 3.7.2) these suspension cultures became highly aggregated, compact and green, but here, many of the aggregates formed roots, whereas previously this happened more rarely.

Aggregates bearing roots were taken from the suspension cultures and used to produce the culture lines examined in the following experiments. The aggregates were removed from the MS-NK liquid medium, and placed on one of two solid media (10 Petri-dishes per medium) upon which they were allowed to proliferate and form callus before being used to initiate immobilized cultures, again in MS-NK. The solid media used were MS-NK and SH (containing 2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K) (Section 2.2.1). The latter was used in the expectation that callus growing from these compact aggregates would be more friable if grown in SH than in MS-NK and would therefore become

immobilized more easily. The callus grown in MS-NK, termed R-MS, was more friable than the aggregates from the suspension cultures grown in the same medium, but it was less friable and darker in colour than the bright green callus which grew on SH medium, termed R-SH.

Cells were taken from each culture line after three weeks growth on solid medium, to initiate immobilized cultures (as described in Section 2.2.4 using five foam blocks) in MS-NK. The callus was not put through an intermediate passage in suspension culture prior to immobilization to avoid the formation of compact aggregates which may not have become immobilized. After three weeks, during which the foam blocks became packed with cells, the R-MS culture lines ranged in colour from creamy to dark green, and roots had formed on the surfaces of the blocks and the suspended aggregates. The R-SH lines (Fig 3.8.1) were all dark green in colour and of a more uniform consistency than the R-MS lines (Fig 3.8.2). More roots appeared to have formed in the R-SH cultures, but these were generally shorter than in the R-MS cultures.

As in previous experiments (Sections 3.4-3.7), the newly immobilized cells were subcultured into fresh medium, and maintained under the conditions described in Section 2.2.3.1 for a further culture passage. Five lines from each group were transferred to MS-NK medium the rest being transferred to MS medium containing only 0.1 mgl^{-1} K (MS-K). These two media were used to determine whether removal of NAA would affect the further development of roots and/or alkaloid production.

The root bearing aggregates left in suspension after the immobilized cells had been subcultured were photographed to illustrate the different appearance of roots from the two sets of culture lines (Fig 3.8.1.ii) and Fig 3.8.2.ii), and were then used to reselect new culture lines. This reselection was performed to find out if the amount of structural differentiation which occurred could be further increased, and because it was expected that the R-MS and R-SH lines would become more heterogeneous with repeated subculture, and may lose any increased ability for organogenesis. The root bearing aggregates from both R-MS and R-SH lines were again placed on SH and MS-NK solid media, and the new, reselected (R-2) lines were termed; R-SH-2SH, R-SH-2MS, R-MS-2SH, and R-MS-2MS. At the same time aggregates which did not bear roots were isolated from suspension cultures maintained in MS-NK, and placed on the same solid media, the resulting "non-rooty" lines being described as NR-SH, and NR- MS.

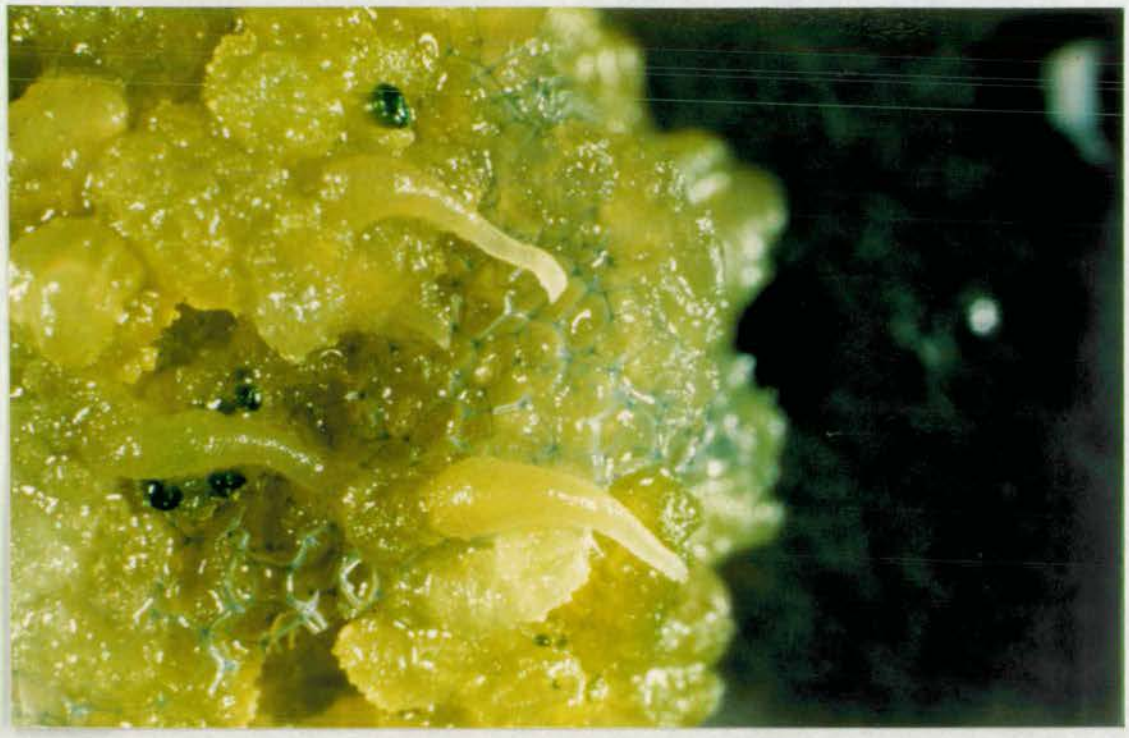
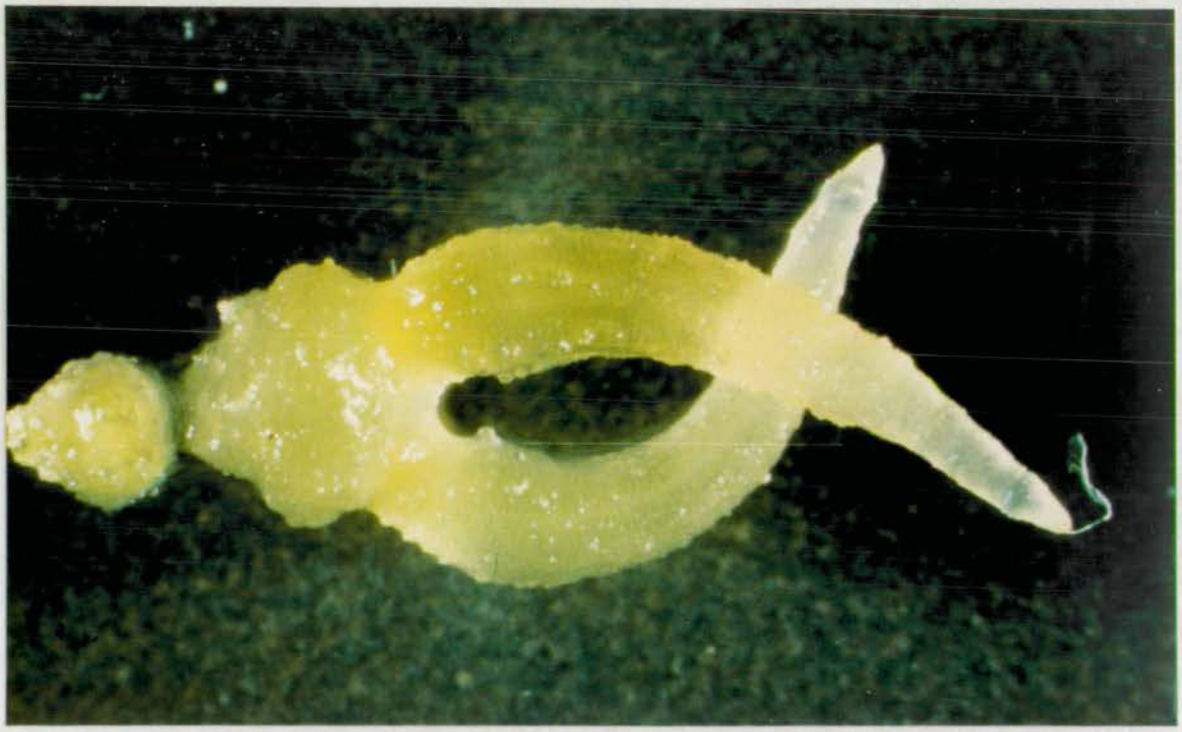


Fig. 3.8.1

i) Roots and shoots formed after one passage in immobilized culture in MS-NK medium by an R-SH culture line of *H. muticus*.



ii) Roots isolated from a suspended aggregate in such a culture, illustrating the typical morphology.

Fig. 3.8.2

i) Roots formed after one passage in immobilized culture in MS-NK medium by an R-MS culture line of *H. muticus*.

ii) An isolated root taken from suspension in such a culture, illustrating the typical morphology.

The immobilized cultures were maintained for 26 days in MS-NK and MS-K before being harvested. First the f. wt. of the cells in each flask was measured, then organogenesis was assessed with the aid of a binocular microscope. Because of the large number of organs formed in the cultures, they were not counted but scored using the following semi-quantitative scale:

Score	No organs per flask
-	0
+	0-5
++	6-20
+++	21-50
++++	over 50

All the cells from each flask were then extracted and analysed by HPLC as described in Sections 2.4.1.1 and 2.4.3, only atropine being detected. The numbers of organs formed per flask, and the alkaloid content of the cells in µg atropine per g f. wt. are shown in Table 3.8.1.

Culture Line Group	Final Culture Medium	Number of Organs per Flask in Blocks Roots	Shoots	Atropine Content $\mu g g^{-1}$ f. wt.
R-MS	MS-K	++	-	28.87
R-MS	MS-K	+	-	ND
R-MS	MS-K	+++	-	ND
R-MS	MS-K	++	-	33.12
R-MS	MS-K	+++	-	51.30
				22.67 +/- 9.99
R-MS	MS-NK	+	-	
R-MS	MS-NK	+	-	5.24
R-MS	MS-NK	+	-	
R-MS	MS-NK	+++	-	2.27
R-MS	MS-NK	++	-	
				3.76 +/- 1.49
R-SH	MS-K	++++	+++	0.77
R-SH	MS-K	+++	++	14.40
R-SH	MS-K	+++	+++	ND
R-SH	MS-K	+++	++	ND
				3.79 +/- 3.54
R-SH	MS-NK	+	-	11.91
R-SH	MS-NK	+++	++++	9.71
R-SH	MS-NK	+++	+++	ND
R-SH	MS-NK	+	-	ND
R-SH	MS-NK	+++	++	ND
				4.32 +/- 2.67

Table 3.8.1

Organogenesis and atropine accumulation by R-MS and R-SH culture lines immobilized in MS-NK medium then incubated in MS-NK or MS-K medium. Organogenesis has been determined semi-quantitatively as described in Section 3.8.1. The means and s.e. of the atropine content for each group of culture lines in each medium are also shown.

Organogenesis

Comparing the number of organs formed in these cultures with the number formed by those grown in MS-NK in the last experiment (Section 3.7.2), it is clear that the isolation and culture on solid media of root bearing aggregates resulted in some lines which had a greater organogenic capacity. Generally the R-SH lines had a greater capacity for organogenesis than the R-MS lines. The R-SH lines produced both roots and shoots, often in large numbers, while the R-MS lines did not form any shoots and produced fewer roots. Again as in the experiment described in Section 3.7.2 the number of roots formed in the absence of auxin was greater than in MS-NK.

Alkaloid Formation

The alkaloid content of the cultures varied considerably, and therefore although the mean alkaloid content of R-MS lines grown in MS-NK was greater than that of the R-MS lines, and the R-SH lines, the difference was not significant. Also the removal of auxin from the medium had no significant effect on the alkaloid content of the cells.

Organogenesis cf. Alkaloid Production

As in the last section (3.7.2) there was no obvious link between alkaloid content and the number of roots and/or shoots they produced. Furthermore, while there was clearly more organogenesis in the R-SH lines, the mean alkaloid content in the R-MS lines was greater, although not significantly different, suggesting an inverse relationship between alkaloid production and root formation.

The R-SH lines grown in MS-NK can be compared with the cultures in the last experiment (Section 3.7.2), which originated from callus grown in SH medium, and were grown in MS-NK for one culture passage in suspension, then two in immobilized culture (the only difference being the additional culture passage in suspension culture for the unselected cultures). The R-SH lines generally produced more roots, and the mean alkaloid content was 3.304 compared with 1.773 $\mu g g^{-1}$ f. wt.; i.e. the alkaloid content of the selected cultures, which generally formed more roots, was higher than that of the stock cultures. Therefore a relationship between root development and atropine production cannot be ruled out, and this was investigated further in the following experiment; where organogenesis was investigated in a fully quantitative manner and related to alkaloid content; and comparisons were made with both the reselected R-2 lines, and the "non-rooty", NR, lines.

Section 3.8.2 FURTHER EXAMINATION OF ALKALOID PRODUCTION IN CULTURE LINES OF *HYOSCYAMUS MUTICUS*

The aim of this experiment was to determine if there was a relationship between organogenesis and alkaloid production in the culture lines which were selected for their different organogenic potentials, whose origin has been described in Section 3.8.1.

The R-SH and R-MS lines were examined again (as in the last section), but as these lines had been through four passages on solid medium, it was feared that they may have become more heterogeneous, or their organogenic characteristics may have been lost. Therefore the reselected (R-2) lines were also examined as they had been through only two culture passages on solid medium, and, being selected from the R-SH and R-MS lines, they may have had an even greater ability to form roots.

In order to determine whether the characteristics of the above lines were due to their origin in root bearing aggregates or to their growth on different solid media, the NR lines, which were derived from non-root bearing aggregates (as described in the last section) were also examined.

The groups of culture lines examined were R-SH, R-MS, R-SH-2SH, R-SH-2MS, R-MS-2SH, R-MS-2MS, NR-SH, and NR-MS (lines from each group were chosen at random, so characteristics of individual R lines cannot be compared between this and the last experiment, only the mean alkaloid content or number of roots produced by the whole group). Approximately 1-2 g f. wt. of cells taken from five culture lines in each of these groups were immobilized, using five foam blocks in 50 ml of MS-NK medium in 250 ml flasks, as described in Section 2.2.4. After a period of four weeks, during which immobilization took place, the immobilized cells from each flask were transferred to fresh MS-NK medium and incubated for four weeks on a rotary shaker under the conditions described in Section 2.2.3.1. The f. wt. of the cells in each flask was then determined prior to examination of the organogenesis which had occurred. This was determined quantitatively by counting all the immobilized and the suspended organs in each flask. The cells in each culture were then extracted (Section 2.4.1.1) and the alkaloid content determined as described in Section 2.4.3. Only atropine was found in the cultures and its concentration in each culture, expressed as $\mu g g^{-1}$ f. wt., is shown, together with the number of roots per g f. wt., in Table 3.8.2. The spent medium from all the cultures in each group was

pooled and extracted as described in Section 2.4.1.2. No atropine was found in any of the medium samples.

Organogenesis

No shoots were formed in any of the culture lines, but, as far as a comparison could be made between the quantitative analysis of this and the semi-quantitative analysis of the last experiment, the general ability of the R-SH and the R-MS lines to form roots had not decreased with the increased number of culture passages.

Again the R-SH lines produced more roots than the R-MS lines, the mean number of roots per g being greater at the 2 % level of significance. And when all the lines from SH solid medium were compared with all those from MS-NK, they had formed significantly more roots, although only at the 10 % level. However, there was no difference between the R-SH-2SH and the R-SH-2MS lines, or between the NR-SH and NR-MS lines.

Reselection from the R-SH and the R-MS lines did not increase the numbers of roots formed, in fact the mean number of roots in the R-SH-2SH and R-SH-2MS lines were less than in the parent lines, although not significantly. Only the R-SH lines formed significantly more roots than the NR lines. However, a number of individual lines from the other R groups produced more than the mean number of roots formed by the NR lines (2.09 +/- s.e. 1.35), which indicates that the crude selection process of isolating lines from root bearing aggregates did produce lines with enhanced organogenic ability, and that growing the isolated aggregates on SH medium probably increased this effect. No difference was noted at the end of the experiment between the MS and the SH lines in the length of the roots which had formed. The appearance of these roots is illustrated in Fig 3.8.3 which shows immobilized cells of the culture line, from the R-SH-2SH group, which produced the most roots. Fig 3.8.4 is a photograph of the typical compact, bright green immobilized cells of one of the NR-SH lines.

Alkaloid Production

As in the last experiment, there was wide variation in atropine content between the culture lines within groups, and there was no significant difference between the R-SH and R-MS lines. However there was a significant difference between the R-SH-2SH and the R-SH-2MS lines, and, more importantly, a difference, at the 1 % level of significance, between the alkaloid content of all the lines grown on SH and MS-NK solid media.

Lines reselected from R-SH and R-MS on SH solid medium had a lower mean alkaloid content, which was similar to that of the NR lines, than those reselected on MS-NK. The R-SH, R-MS and R-SH-2MS lines all had a significantly greater alkaloid content than the NR lines (R-MS-2MS could not be compared as all but one of the cultures derived from these lines in this experiment were lost through contamination). Therefore selection of root bearing aggregates and incubation on solid MS-NK medium resulted in a greater alkaloid content in comparison to cultures derived from non-root bearing aggregates, and to cultures derived from root bearing aggregates grown on SH solid medium.

Organogenesis cf. Alkaloid Production

Since the selection of culture lines from root bearing aggregates was found to increase both organogenesis and alkaloid production, a link between the two was suggested. Yet an inverse relationship was indicated by the fact that more alkaloid was produced by lines grown on MS-NK while those from SH often formed more roots.

To determine if there was any correlation, the amount of atropine per g f. wt. was plotted against the number of roots per g f. wt. in both the MS-NK and the SH lines (Fig 3.8.5). Regression lines, of alkaloid content on the number of roots per g f. wt., were determined for the MS and the SH lines, separately and altogether (Section 2.6). All the regression coefficients were positive, but none were significantly different from zero.

Thus this experiment shows that while some culture lines derived from root bearing aggregates had an increased ability to form roots and/or to accumulate alkaloids, these two variables were not linked. It seems that while deliberately selecting for cells with the ability to differentiate, there was sometimes inadvertent selection of other cells which produced more atropine. The medium on which the aggregates were incubated (MS-NK or SH) also seemed to affect the characteristics of the resulting culture lines. More experiments are required to determine whether it was the basal media (SH and MS), or the different auxins (CPA and 2,4-D cf. NAA), that were responsible for these differences between culture lines of otherwise similar origin.

Culture Line Group	Number of Roots per g f. wt.	Atropine Content µg per g f. wt.
R-MS	1.66	11.56
R-MS	5.03	22.62
R-MS	0.29	2.73
	2.33 +/- 1.41	12.30 +/- 5.75
R-SH	10.56	4.94
R-SH	13.25	6.49
	11.91 +/- 1.34	5.72 +/- 0.77
R-MS-2MS	0.81	6.538
R-MS-2SH	5.21	4.92
R-MS-2SH	1.36	4.34
R-MS-2SH	5.24	ND
R-MS-2SH	1.96	ND
R-MS-2SH	3.23	ND
	3.40 +/- 0.80	1.85 +/- 1.14
R-SH-2SH	10.48	1.30
R-SH-2SH	1.32	ND
R-SH-2SH	4.87	1.30
R-SH-2SH	13.61	2.30
R-SH-2SH	11.24	0.40
	8.30 +/- 2.26	1.07 +/- 0.41
R-SH-2MS	1.36	16.52
R-SH-2MS	0.72	6.98
R-SH-2MS	3.35	11.04
R-SH-2MS	11.53	12.35
R-SH-2MS	2.27	3.14
	3.85 +/- 1.97	10.00 +/- 2.29
NR-MS	0	4.57
NR-MS	0.05	ND
NR-MS	7.67	1.21
	2.57 +/- 2.55	1.93 +/- 1.37
NR-SH	0	2.33
NR-SH	0.09	ND
NR-SH	4.72	0.66
	1.60 +/- 1.56	1.00 +/- 0.70

Table 3.8.2

Organogenesis and alkaloid accumulation by R, R-2, and NR culture lines of *H. muticus* when immobilized and then incubated in MS-NK medium. Values are given for each line together with the means and s.e. of each group of culture lines.

Fig. 3.8.3
The appearance of the culture line of *H. muticus* (from the R-SH-2SH group) which produced the most roots, after two passages in MS-NK medium.

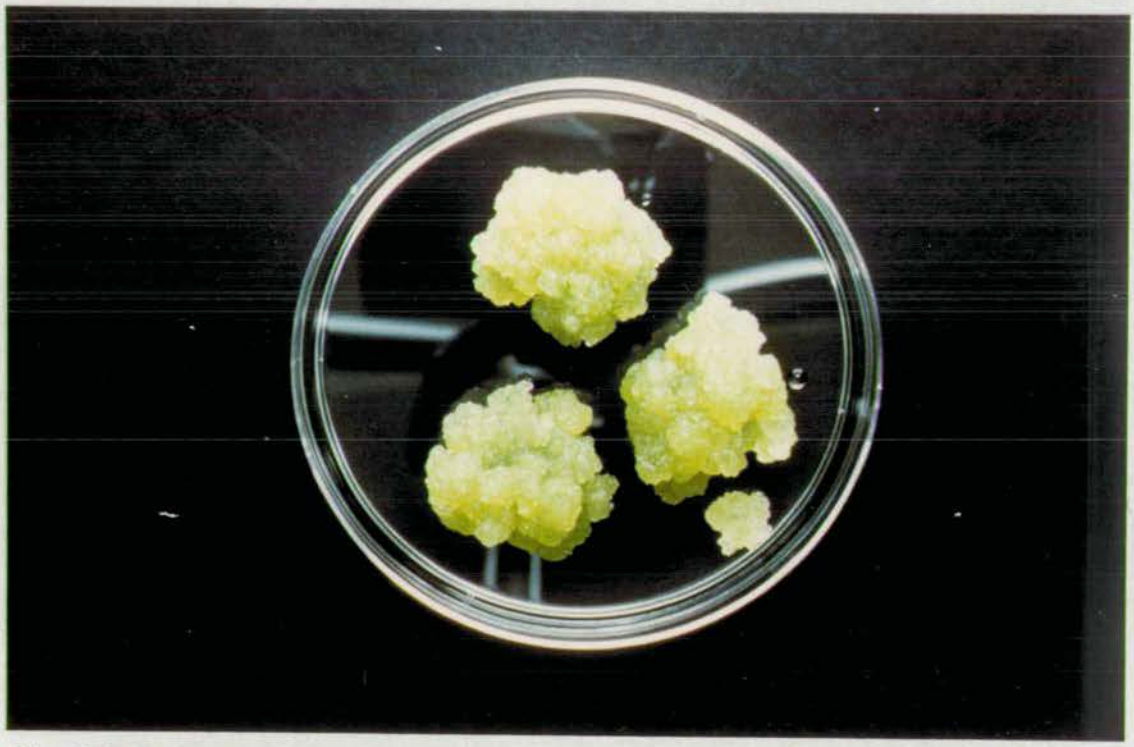
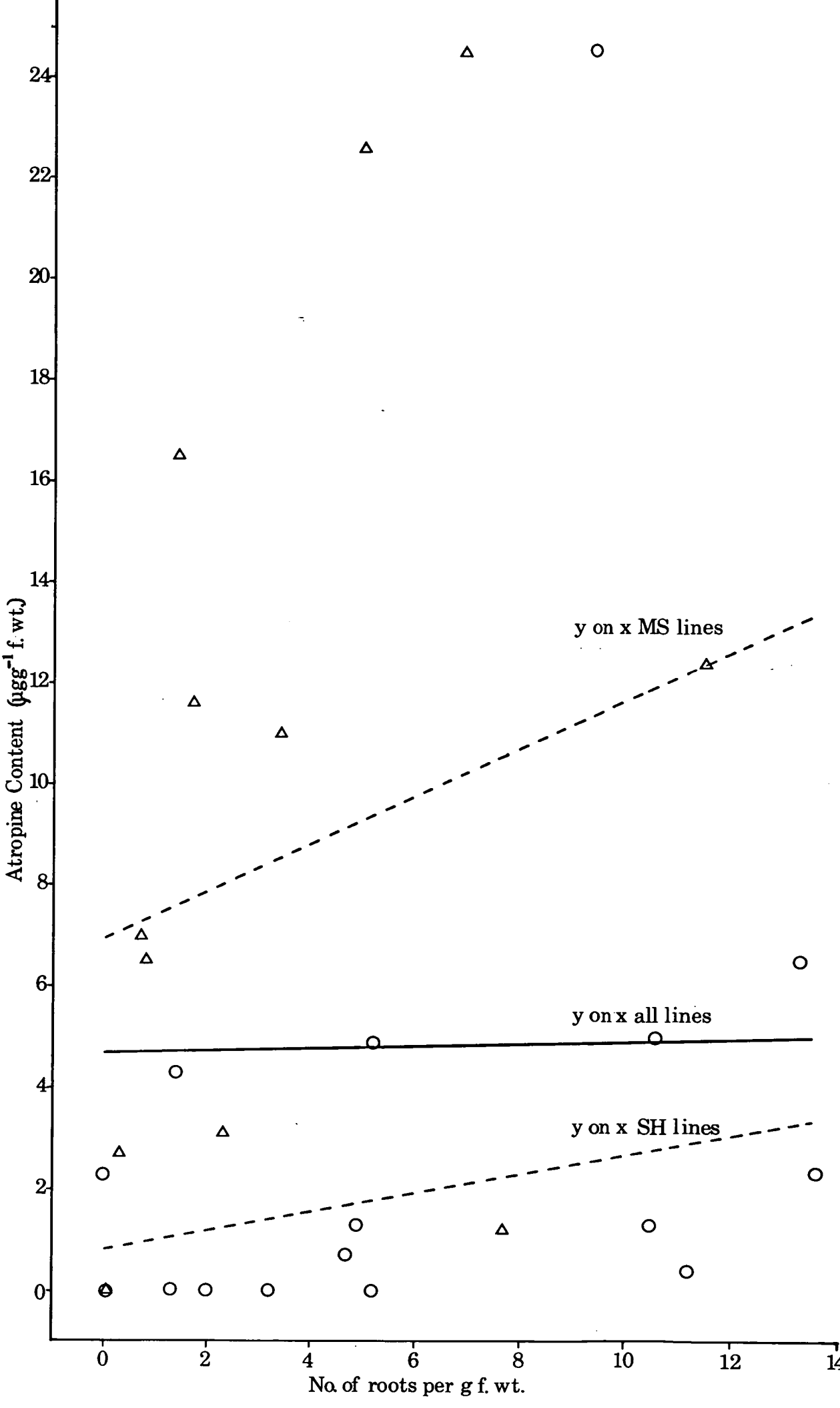


Fig. 3.8.4
The appearance of cells from an NR-SH culture line of *H. muticus* which produced no roots after two culture passages in immobilized culture in MS-NK medium.

Fig. 3.8.5

A scatter diagram showing the relation of the number of roots per g f. wt., x, to the atropine content per g f. wt., y, in culture lines of *H. muticus*. Each point indicates the value of x and y for a single culture line after it had been immobilized and cultured in MS-NK medium. The different symbols represent the solid medium on which the culture lines were previously isolated and maintained; triangles = MS-NK, circles = SH. The fitted regression lines of y on x for the lines grown on SH medium ($y = 0.836 + 0.189x$), on MS-NK medium ($y = 6.88 + 0.481x$), and for all the culture lines ($y = 4.641 + 0.024x$), are also shown.


Atropine Content (μgg⁻¹ f. wt.)
No. of roots per g f. wt.
y on x MS lines
y on x all lines
y on x SH lines
0
2
4
6
8
10
12
14
16
18
20
22
24

Section 3.9 THE PERMEABILIZATION OF SUSPENDED CELLS OF *HYOSCYAMUS MUTICUS*

Tropane alkaloids are accumulated within the cell vacuoles of *Atropa belladonna* both *in vivo* (Verzar-Petri 1973) and *in vitro*. They are released by cultured cells into the medium (West & Mika 1957) but only in small amounts. However, the system envisaged for the production of atropine *in vitro* by immobilized cells involves their long term culture and the continuous, or intermittent, removal of the product from the medium. A method was therefore required of inducing the release of atropine into the medium, which could be used either continuously, or repeatedly after periods of product accumulation by the cells. This method must not be permanently detrimental to the viability of the cells, or their ability to synthesise atropine. In this section attempts are described to determine if such a method can be found for the permeabilization of cultured cells of *H. muticus*.

Since the cell cultures of *H. muticus* used in this study produced only small amounts of atropine, any changes in the levels present in the cells or the medium, which may have been brought about by the treatments used, would have been difficult to detect and determine accurately. Therefore a model system was used; in which attempts were made to cause the release of anthocyanins, which like alkaloids, are stored in the cell vacuole, from suspended cells.

The investigation was carried out with selected culture lines of *H. muticus* which produced large amounts of anthocyanins. These lines were derived from small red-purple groups of cells (about 2-3 mm in diameter) which arose sporadically in the green stock callus cultures. These pigmented cells could be picked out and grown to produce callus lines which appeared completely purple in colour. They were maintained in Petri-dishes on SH medium as described in Section 2.2.3.4, except that on subculturing, only the more strongly pigmented cells were transferred to fresh medium. The suspension cultures used in these experiments were initiated and maintained in SH medium as described in Sections 2.2.3.3 and 2.2.3.4 respectively.

The pigmentation of these cells was assumed to be due to anthocyanins as it had the following characteristics (Hall 1984):

i) The pigmentation was red-purple in colour.

ii) The pigments were located in the vacuole.

iii) The intensity and colour of the pigmentation was pH dependant (changing from red in acid solution to blue green in alkaline solution) and was relatively stable in acid but not in alkaline solutions.

iv) The absorption spectrum of the pigments (in 1 % HCl in methanol) had a major peak at 540 nm, and they were also strongly u.v. absorbent.

The large amount of pigment produced by these cultures and the ease with which it could be determin. ed meant that the effects of the experimental treatments were easier to assess than if the release of atropine had been studied.

The ability of a number of treatments to permeabilize the cells was tested first (Section 3.9.1). Those which appeared to cause the release of anthocyanins were used again in the experiment described in the next section (3.9.2), where the ability of the cells to grow and to accumulate anthocyanins after permeabilization was also assessed. In the final experiment (Section 3.9.3), one treatment was selected and its effect on the cells examined in detail in order to assess its suitability as a permeabilization procedure for general use.

Section 3.9.1 AN ATTEMPT TO FIND A METHOD TO PERMEABILIZE CELLS OF *HYOSCYAMUS MUTICUS*

In this first experiment the purple suspension cultures were exposed to a number of treatments to find which, if any, could cause the release of anthocyanins into the medium. This was determined by measuring the amount of anthocyanin left in the cells after treatment, rather than the amount in the medium, for the following reasons: i) the amount of anthocyanin in the cells could be measured more accurately as it was present at a higher concentration than in the medium, ii) the extract volume could be controlled to give a pigment

concentration which could be determined accurately, iii) replicate anthocyanin extracts could be made from the cells, but not from the medium, iv) the determination of anthocyanin in the medium would require that a known weight of cells was added to the medium, this was not possible particularly in the experiments described in Sections 3.9.2, and 3.9.3 which were performed under sterile conditions, v) the anthocyanins may have been unstable in some of the media, and would therefore have broken down after they were released from the cells.

Since it was important that the permeabilization procedure did not permanently impair cell viability, the effect of these treatments on the cell viability (specific respiration rate) was also determined.

Approximately 6 g f. wt. of fourteen day old suspension culture cells were added to 50 ml of each treatment medium in a 250 ml conical flask which was then sealed with aluminium foil and placed on an orbital skaker (98 rpm, 8 mm amplitude). Four hours later, the anthocyanin content of the cells and the specific respiration rate (K_R) were determined as described in sections 2.5.1 and 2.3.2.1 respectively.
The following treatments were used:

i) SH medium, the control treatment

ii) Distilled water

iii) Increased ionic strength, as described by Tanaka *et al* (1985), magnesium sulphate ($MgSO_4$) was added to SH medium (i.e. in addition to that already present, 1.62 mM) at a concentration of 0.1 M and 0.2 M.

iv) Increased osmotic strength, 20 % w/v mannitol was added to SH medium, this being the concentration previously found to cause incipient plasmolysis of the cells.

v) Alteration of the pH. Here the cells were suspended not in SH medium but in citric acid:disodium hydrogen orthophosphate (citric acid:Na_2HPO_4) buffer (Data for Biochemical Research 1969). Three treatments were used pH 5.6 (i.e. the same as the SH medium), 2.5, and 8.0.

The results are shown in Table 3.9.1.The final anthocyanin content of the cells is shown together with the percentage difference from the control cells, the latter figure indicates the amount of anthocyanin released from the cells.

Cells treated with distilled water and buffer at pH 5.6 had anthocyanin contents which were not significantly different from the controls. Three of the treatments, SH + 0.2 M $MgSO_4$, SH + 20 % mannitol, and buffer at pH 8.0, resulted in an increase of the anthocyanin content per g f. wt. of cells. It is unlikely that this was due to the synthesis of new anthocyanin, but was possibly due to a decrease in the f. wt. of the cells, and thus an increase in the number of cells extracted in each (0.25 g) sample. The anthocyanin content was significantly reduced in those cells which had been exposed to 0.1 M $MgSO_4$ (by 11 %) and to buffer at pH 2.5 (by 60 %). The latter treatment appeared to have damaged the cells, as their K_R was only 25 % of the control value. However, since the cells incubated in water and buffer at pH 8.0 also had a reduced respiration rate, this treatment was investigated further.

In the next experiment the effect of 0.1 M $MgSO_4$ was examined again, but greater attention was paid to the effect of low pH treatment on the cells, a range of pH values between 2.5 and 5.6 being used. To assess the significance of the low respiration rate of cells exposed to low pH buffer, the ability of the cells to grow and to accumulate anthocyanin after treatment was examined.

Treatment medium	Anthocyanin Content of the Cells OD units/0.25g/10ml	Difference from Control Cells (%)	K_R $nmolO_2/ml/min/g$
SH (control)	0.429	0	21.42
Distilled water	0.402	-6.3	10.23
SH + 0.2M $MgSO_4$	0.509	+18.6 (5%)	42.60
SH + 0.1M $MgSO_4$	0.380	-11.4 (5%)	20.18
SH + 20% mannitol	0.570	+32.9 (1%)	25.02
buffer pH 2.5	0.170	-60.4 (0.1%)	5.73
buffer pH 5.8	0.449	+4.7	14.07
buffer pH 8.0	0.603	+40.6 (2%)	8.51

Table 3.9.1
The effect of various treatments on the anthocyanin content and viability of pigmented suspension cultures of *H. muticus*. Where the anthocyanin content of the cells was different from the control, the level of significance is shown in brackets. SH = SH medium, buffer = citric acid:Na_2HPO_4 buffer.

Section 3.9.2 DETERMINATION OF THE EFFECT OF PERMEABILIZATION ON THE SUBSEQUENT GROWTH AND ANTHOCYANIN ACCUMULATION OF CELLS OF *HYOSCYAMUS MUTICUS*

In this experiment, two permeabilization strategies, chosen from those described in the last section, were tested; the addition of 0.1 M $MgSO_4$ to the SH medium, and the use of buffer and SH medium at low pH (i.e. less than pH 5.6). As before, the permeabilizing effect of the treatments was evaluated by determining the anthocyanin content of the cells and comparing this with the amount in the original inoculum, and the effect on cell viability was assessed by determining the specific respiration rate (K_R). However, in this experiment the cells were removed from the treatment media, and incubated in fresh growth medium, in order to determine their ability to grow and accumulate anthocyanin.

Since the cells were to be incubated after treatment, all the manipulations were carried out under sterile conditions with sterile media and equipment (Section 2.2.2). The treatments used are shown in Table 3.9.2. Two control treatments were used; fresh SH medium at pH 5.6, and the spent medium in which the inoculum had been growing. The citric acid:Na_2HPO_4 buffer was made up according to the formula in Data for Biochemical Research (1969). The pH of SH medium was adjusted using M HCl or M KOH, then, after autoclaving, the pH was checked again with sterile indicator paper, and, if necessary, readjusted with sterile M HCl or M KOH. As before, 0.1 M $MgSO_4$ was made up by adding 0.1 $moll^{-1}$ $MgSO_4$ to SH medium in addition to that already present. 50 ml of each treatment medium was sterilized by heat in a 250 ml flask sealed with aluminium foil.

Four 21 day old purple suspension cultures were used as sources of inoculum. They were centrifuged in sterile 50 ml Corning centrifuge tubes, the spent medium was poured away, and about 5 g wet wt. of cells was added to each of the treatment flasks which were then placed on a rotary shaker. Twenty four hours later, the cells were centrifuged again, and a known wet wt. (c. 1 g) of cells was added to fresh SH medium in 250 ml conical flasks, and incubated under the conditions described in Section 2.3.3.1. The pH of the treatment media was measured, and the remaining cells used to determine the viability (Section 2.3.2.1) and anthocyanin content (Section 2.5.1). After 14 days, the cells incubated in fresh medium were harvested and their viability, anthocyanin content, and relative growth index, I_{RG} (Section 2.3.1.4) were determined.

The results of these measurements are shown in Table 3.9.2. The ability of each treatment to permeabilize cells was assessed by comparing the anthocyanin content of the treated cells with that of the inoculum (expressed as percentage difference). The ability of the cells to accumulate anthocyanin and grow after treatment was assessed by comparison with the two controls (SH medium at pH 5.6 and spent medium).

Contrary to the results described in the last section, treatment with 0.1 M $MgSO_4$ did not cause anthocyanin to be released into the medium and was therefore considered to be ineffective as a permeabilization method for cells of *H. muticus*.

Cells treated with SH medium at pH 5.6 and 2.5 clearly lost no anthocyanin, whereas those treated with SH medium at pH 3.5 and 4.0 seem to contain slightly less anthocyanin than the inoculum. One of the three replicate anthocyanin extracts was lost from each of the latter treatments, so significance tests could not be carried out. However, since their anthocyanin content was similar to that of the cells treated with spent medium, and the pH of all the SH media was readjusted by the cells to equal or approach pH 5.6, it is improbable that SH medium originally at pH 4.0 and 3.5 did cause release of anthocyanins.

The cells exposed to buffer at low pH lost anthocyanins which could be seen in the medium, a similar amount being released at pH 4.0 and pH 3.5, more at pH 2.5. Again the values for K_R immediately after treatment suggest that these three treatments were detrimental to the cells, as their K_R was the same or less than that of the inoculum, whereas cells treated with buffer at pH 5.6 or with fresh SH medium had approximately double this respiration rate. This seemed to be borne out by the results obtained after incubation in fresh medium, as the cells treated with buffer at pH 2.5 died. However, the results from cells incubated at pH 3.5, 4.0, and 5.6 are ambiguous. In all three cases, the respiration rate was similar to, or greater than, the fresh SH medium control, but the growth index after treatment with buffer at pH 4.0 and 5.6 was low, while those treated with buffer at pH 3.5 grew as much as the controls. Therefore in the next experiment pH 2.5 was not used, as it killed the cells, but the effect of treatment with buffer at pH 3.0 to 5.6 was examined further.

After being permeabilized by buffer at pH 3.5 and 4.0, the cells contained less anthocyanin than those exposed to control treatments which suggests that their ability to synthesise or to accumulate pigment was impaired. However, the

amounts of anthocyanin accumulated after treatment with SH medium at pH 3.5 and 4.0 (treatments which did not permeabilize the cells) were also less than in the controls. Furthermore while the anthocyanin content of cells was generally lower at the end than at the beginning of the post-treatment culture period, this decrease was relatively small after treatment with buffer at pH 3.5, and there was an increase with buffer at pH 4.0. This shows that permeabilized cultures were able at least to synthesise enough anthocyanin to maintain their post-treatment anthocyanin concentration.

The purple cell cultures when observed microscopically were seen to be composed of a mixture of pigmented and non-pigmented cells. The lower levels of anthocyanin seen in the permeabilized cultures could have been due to a decrease in the amount contained in each of these red cells, or to a reduction in the proportion of pigmented to non-pigmented cells. In order to find out if either of these changes occurred, the cells were again treated with low pH buffer, in an attempt to reproduce the effect recorded here, and to determine its effect on the proportion of pigmented cells in the culture.

Treatment Medium	After Treatment			
	Medium pH	Anthocyanin Content of the Cells OD units/0.25g/10ml	Difference from Inoculum %	K_R $nmolO_2/ml/min/g$
Inoculum		1.340	0	5.37
Spent medium	5.5	1.152	-14.0	5.06
SH at pH 5.6	5.6	1.481	+10.5	10.21
SH at pH 4.0	5.2	1.165	-13.1	10.45
SH at pH 3.5	5.6	1.138	-15.1	10.02
SH at pH 2.5	5.4	1.517	+13.2	11.24
buffer at pH 5.6	6.0	1.652	+23.3	10.37
buffer at pH 4.0	4.3	0.709	-47.1	8.17
buffer at pH 3.5	3.6	0.666	-50.3	5.60
buffer at pH 2.5	2.8	0.256	-80.9	3.42
SH + 0.1M $MgSO_4$	4.2	1.668	+24.5	10.61

Anthocyanin Content of the Cells OD units/0.25g/10ml	After Incubation in Fresh SH Medium: Difference from the Control Cells (%) in SH at pH 5.6	in spent medium	K_R $nmolO_2/ml/min/g$	I_{RG}
1.180	-11.4 (> 10%)	0	4.12	8.543
1.334	0	+13.0	6.93	7.513
0.883	-33.8 (0.2%)	-25.2 (5%)	7.04	14.748
0.881	-33.9 (1%)	-25.3 (5%)	7.19	8.564
1.221	-8.5	+3.5	8.44	10.405
1.043	-21.8 (1%)	-11.6 (> 10%)	10.68	2.560
0.791	-40.7 (1%0)	-33.0 (5%)	10.39	4.002
0.579	-56.6 (0.1%)	-50.9 (1%)	6.18	8.390
-	-	-	0	-0.707
1.063	-20.3 (5%)	-9.9	7.51	7.109

Table 3.9.2

The effect of various treatments on the anthocyanin content and viability of pigmented cells of *H. muticus*, and on their subsequent ability to grow and produce anthocyanins. Where the anthocyanin content was different from the inoculum or from the control, the level of significance has been shown in brackets. (SH = SH medium, buffer = citric acid: Na_2HPO_4 buffer)

Section 3.9.3 EXAMINATION OF THE EFFECT OF CITRIC ACID:DISODIUM HYDROGEN ORTHOPHOSPHATE BUFFER AT LOW PH ON THE SUBSEQUENT GROWTH, ANTHOCYANIN CONTENT, AND PROPORTION OF PIGMENTED CELLS IN SUSPENSION CULTURES OF *HYOSCYAMUS MUTICUS*

In this experiment the effect of citric acid:Na_2HPO_4 buffer at low pH, which had previously been shown to cause the release of anthocyanin from the pigmented cell cultures, without destroying their ability to grow and accumulate anthocyanins, was investigated further. The anthocyanin content and cell viability after treatment, and the relative growth index and anthocyanin content after incubation in fresh medium were measured as before, but the proportion of pigmented to non-pigmented cells in the culture was also determined, to find out if it was affected by the treatment.

Since treatment with buffer at pH 2.5 killed the cells, the lowest pH used here was 3.0. The treatment media were prepared as described in Section 3.9.2 and are listed in Table 3.9.3, SH medium at pH 5.6 being the control treatment.

Two 21 day old purple suspension cultures, A and B, were used as sources of inoculum (see Table 3.9.3). About 5 g wet wt. of cells were treated in 50 ml of each treatment medium for 24 hours as described in the previous section. A known wet wt. (about 1 g) of treated cells was then resuspended in fresh, sterile SH medium (50 ml in a 250 ml flask) and cultured as described in Section 2.2.3.1, and the remaining cells were used to determine the respiration rate (Section 2.3.2.1), the anthocyanin content (Section 2.5.1) and the proportion of pigmented cells (Section 2.5.2). The anthocyanin content of these cells was compared with that of the culture inoculum to assess the permeabilizing effect of the treatments. After 21 days, the cultured cells were harvested, and their relative growth index (Section 2.3.1.3), anthocyanin content, and proportion of pigmented cells were determined. Comparisons were made at this stage between the cells treated with buffer and those treated with SH medium at pH 5.6, in order to assess the recovery of the cells from the treatments. The results are shown in Table 3.9.3, those for the cells exposed to buffer at pH 5.6 being incomplete because of microbial contamination.

Treatment with buffer at pH 3.0 and 3.5 caused release of pigment into the medium, pH 3.0 resulting in a 51 % decrease in the anthocyanin content of the cells, and pH 3.5 in a 38 % decrease. Again, exposure to buffer at low pH was

associated with a lower respiration rate than that of the control cells, and exposure to medium at pH 3.0 caused death of the cells. However, those treated with buffer at pH 3.5 subsequently grew more than the control cells.

Contrary to the results obtained in the last experiment, buffer at pH 4.0 did not cause loss of anthocyanin to the medium. However, when the proportion of red cells was measured after treatment, the protoplast preparations from suspensions treated with buffer at pH 3.0 to 4.0 contained many damaged cells. This could not have been due simply to incubation in the buffer because those treated at pH 5.6 appeared healthy, but must have resulted from the exposure to pH less than 5.6, pH 4.0 having an effect on the cells which was similar to that of pH 3.5 and 3.0, but which was not detectable here in terms of anthocyanin content, viability, or growth index.

After treatment, the cultures treated with buffer at pH 3.5 and 4.0 appeared to contain slightly fewer pigmented cells than the inoculum. However, they contained the same proportion of red cells as the control at the end of the post-treatment incubation period, and those treated at pH 3.0 had the same proportion as the inoculum after treatment, therefore there is no evidence to suggest that the treatment reduces the proportion of pigmented cells in the cultures.

The results show that although treatment with buffer at low pH damaged cells, it did not reduce the proportion of pigmented cells in the cultures, and therefore the lower levels of anthocyanin found in permeabilized cultures after incubation in fresh medium were probably due to a reduced ability of the cells to produce or to accumulate anthocyanin. As in the last experiment, the cells treated with buffer at pH 3.5 were able to maintain the post-treatment anthocyanin concentration better than the control cells. Therefore this method could be a useful permeabilization procedure for cells of *H. muticus* because pigments were accumulated after permeabilization, although not to the original level, and growth and viability were not permanently impaired.

Treatment Medium	Anthocyanin Content OD units/0.25g/10ml	After Treatment Difference from inoculum %	Proportion of Pigmented Cells %	K_R $nmolO_2$ $ml^{-1}min^{-1}g^{-1}$
Inoculum A	1.330		63	4.52
Inoculum B	1.519		58	3.07
SH at pH 5.6 (B)	1.582	+4.1	62	10.59
buffer at pH 5.6 (A)	1.508	+13.4 (5%)	63	8.95
buffer at pH 4.0 (B)	1.408	-7.3	54	10.47
buffer at pH 3.5 (A)	0.828	-37.7 (0.1%)	56	6.01
buffer at pH 3.0 (A)	0.648	-51.3 (0.1%0	67	4.19

After Incubation in Fresh SH Medium			
Anthocyanin Content of the Cells OD units/0.25g/10ml	Difference from SH at pH 5.6 %	Proportion of Pigmented Cells %	I_{RG}
1.175	0	42	11.842
0.921	-21.6 (1%)	51	11.824
0.689	-41.4 (0.1%)	40	13.490
0.029	-97.5 (0.01%)	0	-0.780

Table 3.9.3

The effect of citric acid:Na_2HPO_4 buffer at pH 3.0 to 5.6 on pigmented suspension cultures of *H. muticus*. The letter in brackets after the treatment descriptions indicates the inoculum used. The percentage figures in brackets after some anthocyanin content values are the significance levels for the difference of these values (after treatment) from the inoculum, or (after incubation in fresh medium) from the control (SH at pH 5.6).

SUMMARY

i) An extraction method and an HPLC system for the analysis of the tropane alkaloid content of plant tissue cultures have been developed.

ii) An immobilization method for *A. belladonna* and *H. muticus* has been developed and characterized. Only the immobilized, and not the suspended cells in cultures contained detectable levels of atropine.

iii) Cultures of *H. muticus* produced more alkaloids than those of *A. belladonna*. Therefore subsequent experiments were performed using cultures of *H. muticus*.

iv) Certain alkaloid precursors, when added to the culture medium, increased the amount of alkaloid produced.

v) Cells which did not produce alkaloids were not induced to do so when exposed to nutrient stress.

vi) In immobilized cultures which performed organogenesis, regression analyses found no relationship between the atropine content and the number of roots per g f. wt..

vii) Changing the type and concentration of growth subtances added to the medium can induce morphogenesis, and may affect atropine yield.

viii) Culture lines derived from root bearing aggregates were isolated which produced greater amounts of atropine.

ix) Treatment with reduced external pH may be an effective method of causing the release of secondary products into the medium without adversely affecting the cells.

CHAPTER FOUR

DISCUSSION

Many Solanaceous plants synthesise and accumulate tropane alkaloids. The different organs of these plants accumulate a variety of alkaloids in different amounts, in specific cell types (e.g. epidermal cells) as they mature. Although all parts of the plant may be capable of synthesising these alkaloids they are probably formed mainly in the roots (see Chapter One). Therefore, as both the synthesis and accumulation of tropane alkaloids appear to depend on the cell type, organ, and stage of the growth cycle, they can be considered to be facets of differentiation.

Tropane alkaloid production has been studied in cell cultures derived from a variety of spp, and it has been shown that as for many other secondary metabolites, it is inversely related to growth and is increased by structural differentiation (e.g. Hashimoto *et al* 1986, Lindsey & Yeoman 1983a). Immobilized plant cell cultures are highly aggregated and grow more slowly than suspension cultures, therefore they are likely to produce relatively more secondary metabolites (Lindsey & Yeoman 1983b). The immobilization of cells of *A. belladonna* and *H. muticus*, and the relationship between tropane alkaloid production and growth and differentiation are now discussed.

In order to study secondary metabolism *in vitro* it is necessary to choose highly productive cell cultures, and to use a culture method which is likely to promote secondary metabolite production. The choice of cultures involves the comparison of species which produce the desired substances *in vivo* to find which is able to produce the largest amounts *in vitro* (considered in this section), and cell line or culture line selection (discussed in a later section). Immobilization was chosen as an appropriate culture system. The development of the immobilization system and its effect on the cells is also considered in this section.

The Choice of the Appropriate Species for the Investigation

Zenk *et al* (1977) have stressed the importance of using high yielding culture lines for studies on secondary metabolism. In Section 3.3 it was established that cells of *H. muticus* produced more atropine than cells of *A. belladonna*. This result alone does not show that cell cultures of *H. muticus* always produce more tropane alkaloids than those of *A. belladonna*, particularly since the cultures used in this experiment had been maintained for more than one year, during which they would have become very heterogeneous, and may have changed in their biosynthetic characteristics. But similar results were obtained by Yamada & Hashimoto (1982) using cultures of *A. belladonna* and *H. niger*, only the latter produced alkaloids. One possible reason for *H. muticus* producing more atropine *in vitro* than *A. belladonna* is that the latter species produces less atropine *in vivo*, and higher yielding species give rise to higher yielding cell cultures. However, *A. belladonna* plants contain more alkaloids than those of *H. niger* (Merck Index 1983). Therefore the reason is more likely to be that cultures of *Hyoscyamus* spp are generally able to produce more alkaloids than those of *A. belladonna*.

Studies of secondary metabolite production are best carried out using cells which are relatively high yielding, therefore all the experiments in this study which examined the control of tropane alkaloid production were performed with cultures of *H. muticus*.

Lindsey & Yeoman (1983a) showed that highly aggregated cell cultures produced more alkaloids than friable cultures, and in Table 1.1 it can be seen that callus cultures generally produce more alkaloids than suspension cultures. Therefore immobilized cultures were used in this study because: i) they are highly aggregated, and the degree of aggregation (i.e. the aggregate size) can be controlled; ii) unlike callus culture systems there is good contact of the cells with the medium, therefore their environment can be more fully controlled, and the effects of different media evaluated; and iii) sequential treatments can be imposed with little disturbance of the cells.

The selected procedure, using polyurethane foam as the immobilization matrix, has been shown here (in Section 3.2) to be easily adapted for use with cell cultures of new species. The pore size of the foam was expected to be important for effective immobilization when suspension cultures were very fine or highly aggregated. But the cultures of *H. muticus*, although quite highly aggregated were immobilized effectively in foam of the same pore size as that used for the finer *A. belladonna* cultures, because they contained enough aggregates of an appropiate size to enter the pores and become entrapped in the foam. The foam pore size is therefore likely to be important only when the aggregates in a culture are of uniform size.

The results of Section 3.2.2 show that it was possible to improve the efficiency of the immobilization procedure by increasing the proportion of the biomass in a culture that becomes immobilized. This was achieved by adding more foam blocks (ten rather than four) to the flasks. However, when ten foam blocks were used to immobilize cells in a subsequent experiment (Section 3.3), the cells did not appear to be healthy. This may have been due to inadequate mixing of the cultures caused by the presence of so many foam blocks which could have led to poor aeration, the build-up of toxic substances, or to localized zones of nutrient limitation. Healthy cells were more important than a highly efficient immobilization process, so in following experiments the number of foam blocks per flask was reduced to a level that gave tightly packed cells, which retained their viability, in cultures of both *A. belladonna* and *H. muticus*.

In addition to the simplicity of this immobilization procedure in relation to others, it also has other advantages over the more widely used methods of entrappment in calcium alginate or agarose beads (see Chapter One).

Maintenance of cells in these gels for more than c. 10 days requires the use of a medium which inhibits growth, otherwise the beads break up (Brodelius 1983), and the stability of calcium alginate depends upon a high calcium ion and a low phosphate concentration in the medium. Such a medium could be expected to affect the metabolism of the cells, and the buffering capacity of the medium would be lost or decreased with the reduction of the phosphate concentration. Cells can be maintained in polyurethane blocks for long periods in normal growth medium (e.g. Section 3.4). This allowed the effects of immobilization and growth inhibiting treatments (nutrient limitation and lack of growth substances) to be examined separately.

Alkaloid production has been found to be greater in immobilized compared with suspended cells (Lindsey & Yeoman 1984, Majerus & Pareilleux 1986), and there is some evidence of a similar effect here. In Section 3.3 when the immobilized and suspended cells from each flask were analysed separately, only the immobilized cells contained atropine. Also during the development of the extraction method, callus cultures were found to contain alkaloids (Section 3.1.1.3) but suspension cultures did not (results not shown). In Section 3.4, the effect of immobilization was examined further, suspended and immobilized cultures being compared. This could have provided stronger evidence of whether immobilization *per se* increases tropane alkaloid yield in cultures of *H. muticus*, but none of the cultures in that experiment produced detectable levels of alkaloids. However, immobilized, rather than suspended cultures were used in the following experiments because there is some evidence for immobilization being associated with greater alkaloid yield; and because of the advantages mentioned above of the ability to control the environment of highly aggregated cultures, and to apply sequential treatments without damaging the cells.

The chief problem of an immobilized culture system is that the alkaloids must be released into the medium rather than being accumulated in the vacuole, to allow continuous or intermittent product removal. The attempts made in this study to find a method of inducing the release of secondary metabolites from cells of *H. muticus* are considered in a later section.

Having established the experimental system, the control of tropane alkaloid metabolism was examined. The results of attempts to increase the yield of atropine from immobilized cultures of *H. muticus* are described in the following sections. First, the effects of manipulating various components of the culture medium were examined.

MANIPULATION OF THE CULTURE MEDIUM

Plant cell culture media usually contain the same basic components; macronutrients, micronutrients, vitamins, a carbon source, and growth regulators. The form in which they are added, and the amounts and relative proportions of these components in different media formulations can affect the growth and the secondary metabolism of cell cultures (e.g. Zenk *et al* 1977, Yamada & Fujita 1983). Also, the culture media in general use were designed to support the growth and division of unorganized cells. Since secondary metabolite yield is often inversely proportional to growth, it is likely that these formulations are not optimum for secondary metabolite production. Changes in the nutrient components of the cell culture medium are considered in this section.

The Effect of the Basal Medium

A simple way to examine the effects of the nutrient components of the medium is to use different culture media formulations which have been reported in the literature. In this study the effect on morphogenesis and atropine production in cell cultures of *H. muticus* of three different culture medium formulations was examined. All of these media, SH, MS, and SSM, had previously been used to grow cell cultures, derived from various members of the Solanaceae, which were able to perform organogenesis and to produce tropane alkaloids (see Table 1.1).

In cultures grown in SSM little morphogenesis and no alkaloid production took place. This is probably due to the adverse effect of SSM on the cultures; their growth index was lower and they looked more unhealthy with successive passages in this medium.

Although the highest levels of atropine were accumulated in MS containing 2 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K, there was no clear difference between MS and SH media in their ability to support growth, organogenesis or alkaloid production.

Yields of secondary products from cell cultures can be increased by careful manipulation of the nutrient components of the medium (Yamada & Fujita 1983). But in the specific case of immobilized cultures of *H. muticus*, the potential size of any increase that may be caused by such manipulations is small, because there was no difference in the alkaloid yield from cells grown in the two

quite different culture media, MS and SH.

As mentioned above, it was considered unlikely that culture media designed to support growth would be optimum for secondary metabolism. Therefore the effects of nutrient limiting media, which reduced or inhibited the growth of cell cultures, were examined.

Inhibition of Culture Growth

The theory that primary and secondary metabolic pathways compete for substrates (e.g. amino acids) has been outlined in Chapter One. In this study, the addition of L-phe to the medium of immobilized cells resulted in an increased yield of atropine. The two possible explanations for this are that added L-phe inhibits growth and/or primary metabolism, and stimulates secondary metabolism, including tropane alkaloid production. The second is that the tropic acid synthetic pathway competes with protein synthesis for the precursor L-phe, so adding L-phe to the medium alleviates substrate limitation. Both of these explanations are compatible with the hypothesis that treatments which limit growth, will increase alkaloid production in cell cultures.

In an attempt to tip the proposed balance between primary and secondary metabolism in favour of the latter, and/or to limit growth, cells were grown in media without inorganic nitrogen, or inorganic phosphate or of either of them (Section 3.6). All of these treatment media limited the growth of the immobilized cultures of *H. muticus*, but lack of phosphate and of both nitrogen and phosphate had the greatest effect. Differences in the colouration of the cells in both of these media indicated the increased production of polyphenolics in these cultures, particularly in those lacking only phosphate. However, alkaloids were not detected in any of these treatments.

The effect of nutrient limitation on the production of phenolics (Westcott & Henshaw 1976, Amorim *et al* 1977, Knobloch *et al* 1981), of indole alkaloids (Knobloch *et al* 1981, 1982), and of capsaicin (Lindsey 1985) has been demonstrated. In all of these instances the nutrient limitation caused an increase in the yield of each product rather than an induction of its synthesis (i.e. causing appreciable amounts of the substance to be produced when control cultures contained less than detectable levels). In the experiment described in Section 3.6,

the control cultures did not produce detectable amounts of atropine, so the lack of response in the treated cultures does not rule out the possibility that nutrient limiting media could increase the yield of atropine in cultures which already produced detectable amounts. This view is supported by the stimulation of tropane alkaloid production caused by nitrate and phosphate limitation in transformed root cultures of *Datura stramonium* (Payne *et al* 1987). Therefore future work should include an examination of the effects of the nutrient limiting media used here (MSN, MSP, and MSNP) on cultures which do produce appreciable levels of atropine.

A potentially more successful way of increasing alkaloid levels was to add precursors of the atropine pathway to the medium. The results of this treatment are discussed in the next section.

The Effect of Exogenous Precursors

All of the substances added to the medium of cell cultures of *H. muticus* in the experiments described in Section 3.5 have been shown to be precursors of tropane alkaloids *in vivo* (see Chapter One). But the results show that only L-phe, DL-tropic acid and tropine increased the yield of atropine from immobilized cells (Tables 3.5.2 and 3.5.3).

Atropine production was stimulated by added L-phe and DL-tropic acid which suggests that the supply of tropic acid affects the amount of atropine produced, and that the limitation of yield by this half of the atropine synthetic pathway is probably mediated by the amount of L-phe available as a precursor rather than by the rate of its conversion to tropic acid. This contradicts the hypothesis of Hiraoka *et al* (1973), that the synthesis of tropic acid is repressed in undifferentiated cell cultures. The smaller increase in yield after feeding the cells with DL-tropic acid than with L-phe may be attributable to differences in the response of cells between experiments, despite the similarities of the controls, but it could also be due to incorporation of only one of the isomers of DL-tropic acid into atropine. Thus the effective precursor concentration of 10^{-3} M DL-tropic acid would be half that of 10^{-3} M L-phe.

It is possible that the influence of L-phe, DL-tropic acid, and tropine on the yield of atropine is not direct, as precursors, but indirect, through their effect on

growth and, perhaps, metabolism. Tropine increased the yield of atropine only when it also reduced the growth of immobilized cells; and, although L-phe and DL-tropic acid did not reduce the growth of immobilized cells, they were the only two substances to significantly reduce the growth of suspended cells of *H. muticus*. Therefore the stimulation of yield by these substances may be due to their effect on the activity of the pathway, rather than their ability to act as substrates for it. Similar results were obtained by Tabata *et al* (1972); the alkaloid yield from cells of *Scopolia parviflora* was increased only by tropic acid, which was the precursor that caused the greatest inhibition of growth.

Unexpectedly, L-orn and Na-ppyr both decreased atropine production. A positive, or neutral, but not a negative effect was anticipated, as both substances had previously been found to increase alkaloid yields *in vitro*. L-orn increased the atropine yield in detached leaves (James 1949), and cultured roots (Robinson 1974) of *A. belladonna*, and in cell cultures of *Datura innoxia* (Lindsey & Yeoman 1983a). Hiraoka *et al* (1973) found that Na-ppyr stimulated alkaloid production in cultures of *Datura innoxia*, but L-phe did not.

All of the replicate cultures from the experiments described in Section 3.5 were pooled together prior to analysis of their alkaloid content. Future work should include a similar experiment to re-examine the effects of the precursors, in which the replicates are analysed separately, so that any variation in the response of cultures to each precursor can be seen. Once the effect of added precursors on the amount of alkaloid accumulated by cells has been defined, their fate in the cell could be further examined by feeding radioactively-labelled precursors. Such work would show whether the precursors are actually incorporated into tropane alkaloids, or have an indirect effect on alkaloid production. If they are incorporated, the experiments could also provide information about the limiting steps of the pathway, and, if combined with other treatments which affect the yield, they could indicate how alkaloid metabolism is controlled, as the fate of the labelled precursors, and their rate of uptake and incorporation may vary with the culture conditions.

The results of experiments to examine the effects of; the nature of the basal medium, the removal of certain macronutrients from the medium, and the addition of alkaloid precursors to the medium, have been considered. The nature and concentration of the growth substances added to the medium are also known to effect secondary metabolism (Mantell & Smith 1983). Their effect on tropane alkaloid metabolism is considered within the next main section, which concerns differentiation in immobilized cultures of *H. muticus*.

The production of certain secondary metabolites *in vitro* has been shown to be influenced by, or to depend on, differentiation (Yeoman *et al* 1980, Yeoman *et al* 1982), where differentiation refers to aggregation or the formation of recognisable plant tissues or organs by the cultures. However, "differentiation" is defined as:

> "A process of change, both structural and functional, in cells, tissues, or organs, during development; resulting in the appearance, where they were previously lacking, of structures and functions that characterize the different kinds of cells etc. of different parts of the adult, or of different stages during the life cycle." (Penguin Dictionary of Biology 1980)

One of the characteristics of secondary products is that they are only produced or accumulated by certain cells or tissues at certain stages of the life cycle. Therefore cells which produce secondary products can be considered to be "differentiated". In this treatise, to avoid ambiguity, organogenesis has been more precisely defined as structural differentiation, and changes in the shape or metabolism of the cells themselves, including the synthesis of secondary products (in this case tropane alkaloids), is termed cytodifferentiation.

An examination of the proposed relationship between cyto- and structural differentiation is difficult because the treatments which induce structural differentiation (manipulation of the growth substances added to the medium) probably have independant effects on the metabolism of the cells. It is important to recognise that organogenesis and secondary metabolite production may be independant responses to such treatments, rather than closely linked, sequential markers or stages in the differentiation of a certain tissue type, or organ.

The relationship between organogenesis and alkaloid production, and the influence of growth substances added to the medium are now discussed.

Structural Differentiation and Tropane Alkaloid Production

Root formation has been found to be necessary for tropane alkaloid production *in vitro* (West & Mika 1957, RajBhandary *et al* 1969, Endo & Yamada 1985), or to increase the levels that are produced (Thomas & Street

1970). But in this study unorganised cultures of both *A. belladonna* and *H. muticus* were able to produce alkaloids; and analyses of the regression of atropine content on the number of roots per g f. wt. in immobilized cultures of *H. muticus* showed that there was no relationship between them (Figs 3.7.8 and 3.8.5); i.e. in immobilized cultures of *H. muticus*, alkaloid yield does not depend on structural differentiation.

The results of Section 3.8.2 in particular provide good evidence for this conclusion. The experiment involved callus lines with varying abilities to form roots (which were derived from root bearing aggregates in suspension cultures growing in MS-NK medium). Therefore different media, which could have had independent effects on alkaloid metabolism, were not required to induce different amounts of structural differentiation. The alkaloid content of these lines, when immobilized in MSNK medium, varied between the culture lines and seemed to be affected by the solid medium on which the root bearing aggregates were grown. These were SH (containing 2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4,D, and 0.1 mgl^{-1} K) and MS-NK (containing 1.0 mgl^{-1} NAA, and 0.1 mgl^{-1} K). When NR-SH and NR-MS lines (derived from non-root bearing aggregates grown on MS-NK and SH media) were compared, no difference was found in the number of roots or in the amount of alkaloids formed, but when all of the isolated lines (R, R-2, and NR) grown on the two media were compared, the lines grown on SH medium formed more roots, but produced less atropine than those lines grown on MS-NK medium. This suggests that alkaloid production and root formation in these cultures are controlled differently.

Previously the alkaloid content of suspension cultures of *A. belladonna* has been shown to vary with the frequency of root formation (Thomas & Street 1970). But the results of Tabata *et al* (1972), Dhoot & Henshaw (1977), and Eapen *et al* (1978a) showed that the total alkaloid content of unorganised cell cultures was the similar to that of cultures which performed organogenesis. However, in all of these three reports, although structural differentiation did not affect the alkaloid yield, it did affect the proportions of the different tropane alkaloids that were accumulated. Dhoot & Henshaw (1977) found that cultures of *H. niger* lost their ability to perform organogenesis and ceased to produce hyoscyamine at the 14th serial subculture, whereas their total alkaloid content remained the same until after the 35th subculture. In callus cultures of *Scopolia parviflora* which produced roots, the total alkaloid content of the unorganised tissue and of the roots was the same, but, whereas the roots had a similar alkaloid pattern to that of roots *in vivo*, the callus tissue contained a smaller

proportion of hyoscyamine and scopolamine (Tabata *et al* 1972). Scopolamine production seemed to be related to root formation in cultures of *Datura innoxia* (Hiraoka & Tabata 1974) and of *H. niger* (Hashimoto & Yamada 1983); but in unorganised callus of *A. belladonna* scopolamine was the predominant alkaloid rather than hyoscyamine as *in vivo* (Eapen *et al* 1978b).

In this study the presence of minor alkaloids in cell cultures was not determined. Of the major alkaloids looked for, scopolamine, hyoscyamine-N-oxide, and hyoscyamine, only the latter was detected. The atropine concentration was similar in unorganized and organized cultures. Therefore, if atropine was not the predominant alkaloid in the unorganized cultures, they must have produced more total alkaloids than the organized cultures. Since this is very unlikely, it is considered that in both root forming and unorganized cultures of *H. muticus*, only atropine was formed, or it was the predominant alkaloid.

The results show that organogenesis is not essential for atropine production in immobilized cultures of *H. muticus*, and that there is no direct correlation between alkaloid content and root formation. It is possible that since even the unorganized cultures are in a highly aggregated state, due to immobilization, that further organization to form roots had little or no effect. An examination of the effect of root formation on the alkaloid yield of immobilized and suspended cultures would show whether the high degree of aggregation in immobilized cultures masks any effect that organogenesis may have on atropine production.

However, the results do indicate that growth substances may be controlling factors and this is dealt with in the following section, after which the role of structural differentiation will be considered further.

Plant Growth Substances and Tropane Alkaloid Production

Although the experiments described in Sections 3.7 and 3.8 were designed to find out if there was a relationship between structural organization and alkaloid production, they indicate instead that the growth substances added to the culture medium affect secondary metabolism. First the results of the experiment described in Section 3.7.2 are considered.

The cultures were grown in six primary media SH-CDK, SH-NK, MS-CDK, MS-NK, SSM-CDK, and SSM-NK then immobilized cells from each primary medium were transferred to the same medium (containing auxin and cytokinin), or into the same basal medium containing kinetin at the same, or at 10 times, the original concentration but no auxin; e.g. SH-CDK, SH-K, and SH-10K (the complete list of these media is shown in Table 3.7.2). Comparing the alkaloid contents of cultures in each group of three secondary treatments (see example above), the highest atropine levels were accumulated in the presence of auxin while the number of roots per g f. wt. was greatest in the presence of only 0.1 mgl^{-1} K.

The Effect of Kinetin

The effect on the atropine yield of increasing the kinetin concentration (in the absence of auxin) varied between the groups of secondary media. However, more experiments are required to find out if kinetin can influence atropine production in these particular cultures, and to determine the nature of such an effect.

Relatively little attention has been paid previously to the effect of kinetin on tropane alkaloid metabolism *in vitro*, and the results have varied between the different studies. Sharma & Khanna (1982) found the highest levels of atropine in cells of *A. belladonna* grown in medium containing $1mgl^{-1}$ K, compared with cells grown in the presence of auxin, or without growth substances, but increasing the concentration of kinetin reduced the amount of alkaloid produced. Similarly Szoke *et al* (1982) found that (in the prescence of 1 mgl^{-1} 2,4-D) raising the concentration of K from 1-5 mgl^{-1} progressively suppressed the alkaloid yield from cells of *Datura innoxia*. However, Tabata *et al* (1971) found that increasing the kinetin concentration increased the amount of nicotine produced by cultures of *Nicotiana tabacum*. Therefore it is conceivable that added cytokinin will influence tropane alkaloid production by cultures of *H. muticus*, but the effect of e.g. an increase in its concentration could be positive or negative and will depend almost entirely on the cells involved and their pre-culture history.

The Effect of Auxin

Although, in the experiment described in Section 3.7, the presence of auxin was associated with an apparently greater yield of atropine, in the results shown in

Table 3.8.1, the presence or absence of auxin had no significant effect. The reason for this difference in response between experiments is probably the different nature and culture history of the cells used. In Section 3.7.2, stock cultures of *H. muticus*, grown on SH medium, were used; and in Section 3.8.1, the culture lines were derived from root bearing aggregates grown on SH or MS-NK solid media.

Auxin has previously been shown to affect the production of tropane alkaloids *in vitro* in different ways; its presence in the medium has been responsible for increasing and decreasing yields (in different systems). In the present study the yield of atropine was reduced by the removal of auxin from the medium (Table 3.7.2). A similar relationship was seen by Tabata *et al* (1972) in callus culture of *Scopolia parviflora*, where increasing the 2,4-D concentration from 10^{-7} to 10^{-4} M increased the yield of tropane alkaloids. Szoke *et al* (1982) found that both increasing and decreasing the concentration of 2,4-D added to the medium from 2 mgl^{-1} stimulated the tropane alkaloid yield in cultures of *Datura innoxia*.

Where the removal of auxin from the medium has been found to stimulate alkaloid production, this has often been attributed to the concomitant induction of organogenesis. However, in this study, the conditions which were optimal for structural differentiation, i.e. medium without auxin, were not the best for alkaloid production. Furthermore, although the alkaloid content in cultures of *A. belladonna* grown in medium without auxin varied with the frequency of root formation, non-root bearing aggregates were shown to contain alkaloids, while in medium containing auxin, the cultures did not produce any alkaloids (Thomas & Street 1970). And, while differentiated cultures of *Nicotiana tabacum* produced more nicotine than non-differentiated cultures, a much greater increase in nicotine content was caused by the removal of IAA from the medium than by organogenesis: in the presence of 0.2 mgl^{-1} K, the increase in yield on the removal of 2 mgl^{-1} IAA from the medium was 19 fold; while in medium containing 2 mgl^{-1} K, and no auxin, the difference in nicotine content beween cultures which did and which did not perform organogenesis was only 2 fold. Also in cultures of excised roots of a number of species of Solanaceae, the yield of tropane alkaloids was greater in the absence of auxin (Hashimoto & Yamada 1986).

In addition to the immediate effect of auxin indicated by these results, i.e. the effect of the amount added to the medium in which the cells were growing

prior to analysis, it also seems to have affected the nature of the culture lines which were isolated and examined as described in Section 3.8.

Cell suspension cultures of *H. muticus* in MS-NK medium (i.e. containing 1 mgl^{-1} NAA, and 0.1 mgl^{-1} K) initiated from callus cultures on MS-CDK medium (i.e. containing 2.0 mgl^{-1} CPA, 0.5 mgl^{-1} 2,4-D, and 0.1 mgl^{-1} K) produced numerous roots. The root bearing aggregates were isolated and grown on one of two solid media, SH-CDK and MS-NK. The culture lines derived on these media were termed R-SH and R-MS respectively. When in immobilized culture in MS-NK medium, the R-SH lines generally produced more roots and less alkaloids than the R-MS lines.

As they originated from the same root forming suspension cultures the only difference in the treatment of these groups of lines was the two solid media on which they were grown. Their different characteristics are not likely to be due to the basal media, MS and SH, because the results in Section 3.7 showed that these had similar effects. Therefore it was probably the different growth substance combinations in the media which affected the subsequent ability of the R-MS and R-SH lines to form roots and to produce alkaloids. This hypothesis could be tested by comparing similar culture lines which had been grown on MS-NK, MS-CDK, SH-NK, and SH-CDK solid media.

The way in which auxin may influence secondary metabolism *in vitro* is difficult to determine. In some cases, there is an indication that it may be mediated by its effect on growth. IBA was neccessary for good growth of root cultures of *Atropa, Datura*, and *Duboisia* species, its removal from the medium was associated with increased alkaloid levels (Hashimoto & Yamada 1986). An increase in the concentration of 2,4-D, or a decrease in the concentration of IAA or NAA, were associated with decreased growth and increased atropine production by callus cultures of *A. belladonna* (Sharma & Khanna 1982).

However, growth was not affected by the different growth substance combinations used in the present study (Table 3.7.1), which suggests that any effect they may have on secondary metabolism is not mediated by growth. Simola & Saponen (1971) observed that although there was more growth in cultures of *A. belladonna* grown in the absence of auxin, those grown in the presence of NAA had a higher protein content, and the activity of certain primary metabolic enzymes was greater. Phillips & Henshaw (1977) found that

2,4-D inhibited the accumulation of phenolics by nitrogen and phosphate limited cells of *Acer pseudoplatanus*, and stimulated the turnover of protein. Thus the auxin added to the culture medium has been shown to affect the metabolism of cells, in addition to, or without affecting their growth.

To summarise, there is an indication that the yield of atropine from immobilized cultures of *H. muticus* could be increased by finding the optimum mixture of growth substances for addition to the growth medium. Important factors are likely to be the concentration, and type of auxin, and the relative concentration of kinetin, both in the culture period prior to analysis and in the preceding culture passages (i.e. the culture history). Alkaloid yields may be increased by adopting a two stage culture system with respect to growth substance supplementation of the medium. The sequential treatments may play a similar role to those causing embryogenesis in cell cultures; where the primary medium is necessary for the "induction" of the embryos, the secondary medium for their "maturation" (Ammirato 1983).

Returning to the aims of this project, increasing the alkaloid yield from cultured cells by manipulation of the growth substances added to the medium would be appropriate to the development of a commercial system for the production of atropine *in vitro*, since only increased yield is important. However, controlling the alkaloid production in cell cultures by such manipulations cannot provide any information about the control of alkaloid metabolism. The type and the amount of growth substances added to the culture medium has been shown to influence the production of various secondary products, in numerous reports (reviewed in Mantell & Smith 1983). The effects vary with each culture system, so, any treatment that may be developed which stimulates tropane alkaloid production *in vitro* will only do so in those particular cultures. The different results obtained with different culture systems suggest that the decreases or increases in alkaloid production are not direct responses to the manipulations of the growth substances, which could be expected to be relatively consistent, but are more likely to be an arbitrary reaction to the stress which has been imposed by treatment with artificial growth substances, at far from physiological levels.

Further Consideration of Alkaloid Yield and Organogenesis

An examination of Table 1.1 shows that, while there are exceptions, root cultures generally produced more alkaloids than unorganised callus or suspension

cultures. The two basic explanations for this observation are i) that within these structures the environment is similar to that in the whole plant. Thus, if auxin does in fact control tropane alkaloid metabolism *in vivo*, it will be present in the appropriate amount, of the correct type, and with the gradients required for the production of "normal" levels of alkaloids. Also in the organised structure the balance between primary and secondary metabolism may be different to that of unorganised cell cultures. And ii) in root cultures there would be less likelihood that cells would undergo somaclonal variation and therefore they are likely to retain full totipotency, including the ability to express tropane alkaloid metabolism. Cells in unorganised culture often go through genetic or epigenetic changes which leave them unable or less capable of expressing certain characters.

However, this does not account for the results of this study where experiments designed to detect a relationship between organogenesis and tropane alkaloid production found none. It has already been proposed that the high degree of organization in immobilized cultures obviates the need for root formation to increase atropine yield, But is is possi. ble that differences in the media used for unorganized cultures and excised root cultures are responsible. Unorganized cultures, including the cells used in this study, were subjected to relatively high concentrations of growth substances for a long period of time prior to these particular experiments, and these may have influenced the characteristics of the cultures lines. In contrast, root cultures are generally grown in the absence of, or with small concentrations of growth substances, and so have been subjected to less stressful, less non-physiological conditions than callus and suspension cultures.

The results of this study have shown that the culture medium can have relatively long-lasting affects on the nature of culture lines. The R-MS and R-SH culture lines were derived from the same suspension cultures, and, at the time of analysis had been grown in MS-NK medium for two 4 week culture passages, yet lines initiated on MS-NK and SH solid media had different characteristics in terms of organogenesis and alkaloid production.

In the experiment in Section 3.7.2, the maximum effect of the removal of auxin was reduction of the yield by two thirds. Feeding certain precursors increased the yield two fold. Yet the mean yield from the R lines was six times greater than that from the NR lines. Therefore a greater increase in the yield of atropine was achieved by indirect selection of culture lines than by the manipulation of the environment. The cultures have also varied in their response to added precursors (Section 3.5) and growth substances (Sections 3.7.2, and 3.8). Similarly Mano *et al* (1986) found that different transformed root clones varied in their response to basal media, the nitrogen source, and the medium pH. Thus, although treatments can be developed which increase the alkaloid yield, they tell us little about how secondary metabolism is regulated, as the reaction of the culture lines to the treatments is of greater importance. More information about the control of secondary metabolism may be obtained by performing detailed comparisons of high and low yielding culture lines.

Cell or culture line selection is a powerful means of increasing secondary product yields *in vitro*, it is considered further in the following section.

The importance of the selection of high producing lines when developing a system for secondary metabolite production *in vitro* has been stressed repeatedly (e.g. Zenk 1978, Yamada & Fujita 1983, Mano *et al* 1986). In this study, crude selection by the isolation and subculture of highly pigmented portions of callus was used to generate high anthocyanin producing culture lines of *H. muticus* (Section 3.9); and simple indirect selection, i.e. the isolation and culture of root bearing aggregates gave high hyoscyamine producing (R) lines (Section 3.8). The reselected R-2 lines did not accumulate more atropine than the R lines, however, since only a relatively small number of lines were originally isolated, and the reselection was performed on only a small scale, the increases in yield that could be achieved by isolating culture lines in this way are potentially greater.

The original aim of the procedure described in Section 3.8.1 was to isolate cell lines with an increased ability to perform organogenesis. A number of lines were isolated which produced more roots than the NR lines, although the only group of lines to produce significantly more roots than the NR lines was the R-SH group.

Alkaloid production was not related to the number of roots formed in cultures of *H. muticus*, nevertheless the yield was increased by isolating and culturing root bearing aggregates, the R-SH, R-MS, and RSH-2-MS lines had a significantly greater atropine content than the NR lines. It is possible that the selected cells, which were capable of structural differentiation, were those cells which had retained their totipotency, i.e those cells which had not gone through genetic or epigenetic changes during repeated subculture. Such cells are more likely to have a functional mechanism for the regulation and production of tropane alkaloids than cells which have changed while in culture. There are previous results that support this explanation: Hiraoka & Tabata (1974) regenerated *Datura innoxia* plants, 82 % of which were diploid, despite the fact that, in the suspension cultures from which the plants were derived, only 32 % of the dividing cells were diploid.

Of equal importance to the selection of high producing cell lines, is their stability during prolonged maintenance *in vitro*. Cultures of single cell origin can rapidly become heterogeneous, and produce less of the desired secondary product. This occurred in single cell clones of *H. muticus* selected for their high scopolamine production by Fankhauser *et al* (1986). The scopolamine content of

the most highly productive clones decreased from 0.5 to 5 x 10^{-5} % dry wt. between the fourth and tenth culture passages after isolation. Attempts to stabilize this degeneration by recurrent selection of high producing subcultures were unsuccessful. However, in other cases, the repetition of selection did recover high producing cell lines (Deus-Neumann & Zenk 1984a, Sato & Yamada 1984); and repeated single cell (Sato & Yamada 1984) and cell aggregate cloning (Yamada 1984) has resulted in the isolation of stable culture lines.

In this study it was not possible to examine the stability of the R and R-2 lines, but the mean alkaloid content of the R lines did not fall during the three culture passages between the experiments reported in Section 3.8.1, and 3.8.2. However, it would be necessary to carefully monitor the stability of any lines used in future experiments. These lines may prove to be more stable than single cell clones, because it is probably the stress imposed during the isolation of the latter which causes their instability.

In this project attention was focussed on the control of alkaloid production by manipulation of the environment, therefore direct selection of culture lines for alkaloid content was not performed. However, it may be interesting in future to compare alkaloid production in both directly and indirectly selected lines, and their ability to perform organogenesis. If the directly selected, high yielding lines were found to produce more roots than control cultures, this would support the theory outlined above, that selection for morphogenetic ability also selected for increased alklaoid production, because it isolated totipotent cells.

The role of the lines already isolated, or of new lines, which may be selected by the procedure described in Section 3.8.1, or by direct selection, will be discussed in the section headed Future Work.

THE RELEASE OF ALKALOID INTO THE MEDIUM

Spontaneous Release

Few of the reports on tropane alkaloid production in cell cultures mention whether alkaloids were looked for in the medium. But since, where this was examined, the amounts found were always small, the lack of information suggests that the medium was analysed more often and the results were negative. This theory is supported by the fact that all the recorded instances of tropane alkaloid release involve root cultures rather than unorganised cultures, except for the *Datura tatula* callus which released 2-18 % of the total alkaloids produced into the medium (Hiraoka *et al* 1973). Furthermore, Hashimoto & Yamada (1983) found that suspension cultures of *H. niger* did not release hyoscyamine or scopolamine into the medium, yet root cultures derived from these suspension cultures always released scopolamine, and sometimes hyoscyamine. Cultured roots themselves do not always release alkaloids. Mitra (1972) found no alkaloids in the medium of cultured roots of *A. belladonna*; West & Mika (1957) did, but only in very small amounts. Rhodes' group looked for the release of alkaloids from transformed roots of various Solanaceae. While "hairy" roots of *Nicotiana rustica* released nicotine (Hamill *et al* 1986, Rhodes *et al* 1986), transformed roots of *Datura stramonium* released less than 1 % of the tropane alkaloids they produced into the medium (Payne *et al* 1987).

Since roots *in vivo* probably synthesise much of the atropine produced by the plant and export it to the aerial parts, it was hoped that the differentiation of roots *in vitro* may be associated with the release of alkaloids into the medium. Also it was possible that immobilization *per se* would cause release, as it did when cells of *Mucuna pruriens* were entrapped in calcium alginate (Wichers *et al* 1983). However immobilized cultures of *H. muticus*, whether unorganized or showing extensive root formation, did not release atropine into the medium (Sections 3.3 and 3.8.2).

Release of the product into the medium is crucial for the proposed industrial immobilized systems. Where the cultures normally accumulate the product intracellularly, as in the system considered here, there are two basic strategies for obtaining its release; the selection of a culture line which does not accumulate the product intracellularly; and manipulation of the environment of the existing cultures to cause release of the product, i.e. to permeabilize the cells. An excreting culture line would be preferable, as a permeabilization step would

increase the complexity (and cost) of the system, and would probably be detrimental to the cells, at least in the long term.

Culture lines of *Thalictrum minus* (Nakagawa *et al* 1984) and *Coptis japonica* (Sato & Yamada 1984) which released berberine were isolated with apparent ease. However, it is perhaps significant that berberine was the alkaloid exported in both cases, since, despite their intrinsic value, there are no other reports of the existence of excreting culture lines, which points to a lack of success as much as to a lack of effort towards this objective.

The principle aim of this study was to study the production of tropane alkaloids *in vitro*, and therefore the screening of cell lines for one which excretes tropane alkaloids was not feasible. However, since product release is of such importance, a number of permeabilization methods were assessed in the hope of finding a treatment which could induce the release of secondary products from the vacuoles of cells of *H. muticus* without reducing their viability or productivity.

Permeabilization

Physical methods of permeabilization (pH, ionic strength, osmotic pressure) were used as they were considered less likely than chemical treatments to be irreversible or detrimental to the cells. They were examined using the model system of anthocyanin release from suspended cells of *H. muticus* (Section 3.9).

Treatment with medium of high ionic strength did not permeabilize the cells in this study, yet Tanaka *et al* (1985) found it to be a successful permeabilization agent which did not affect cell viability. The reason for this is probably that they measured the release of an enzyme (5-phosphodiesterase) from the cytoplasm, whereas in the model system described in Section 3.9, release from the vacuole was required. This involves permeabilization of the tonoplast as well as the plasmalemma, and the reversal of what is probably active accumulation of anthocyanins in the vacuole.

The results presented here show that only reduction of the external pH effectively permeabilized the cells of *H. muticus* and they were subsequently able to grow and produce anthocyanins. The levels accumulated after permeabilization were lower than in control treatments, but it may be possible to

overcome this by modifying the treatment.

The simplest modification would be to shorten the treatment time. Cells were exposed to the low pH buffer overnight, but treatment for a matter of hours would probably suffice. The treatment medium itself could be modified. The concentration or the nature of the buffer could be changed; or it could be combined with growth medium or a more simple mixture of salts and sucrose in order to protect the cells from undue stress. Finally the cells could be permeabilized by raising the intracellular pH (rather than lowering the extracellular pH) using methylamine or FCCP (carbonylcyanide-p-triflormethoxy-phenylhydrazone) (Kurkdjian 1982). These modifications should be made with reference to both the model system, i.e. cultures which accumulate large amounts of anthocyanins, and cultures of *H. muticus* which produce alkaloids. The former system has the advantage of ease of analysis, and the large amounts of anthocyanins which are produced make quantitative comparisons accurate, but it is essential to ascertain whether lowering the external pH causes release of alkaloids from the vacuoles in addition to anthocyanins.

It is reasonable to suppose that the low pH treatment could cause the release of alkaloids into the medium, as it accords with the proposed mechanisms for alkaloid accumulation into the vacuole; the ion trapping model, and active carrier mediated transport.

The ion trapping model (Kurkdjian 1982, Renaudin & Guern 1982, Renaudin *et al* 1985) relies on the alkaloids having a neutral, lipophilic form which freely diffuses through membranes. Thus they accumulate in the acidic vacuoles as their non-diffusible cations. Experiments showed that this model is valid for the uptake of exogenous indole alkaloids in *Catharanthus roseus* (Renaudin & Guern 1982, Renaudin *et al* 1985) and of exogenous nicotine in *Nicotiana tabacum* and *Acer pseudoplatanus* (Kurkdjian 1982). The pH gradient between the vacuole and the medium controlled the extent of accumulation of the exogenous alkaloids into the vacuole, and acidification of the medium led to their efflux. The model could not fully account for the accumulation ratios of endogenous alkaloids e.g. that for endogenous ajmalicine was tenfold that of exogenous ajmalicine. This was explained by the (slowly reversible or irreversible) binding of alkaloids to other components in the vacuole, possibly phenolics, since active uptake was not compatible with the data of Kurkdjian (1982).

However, cells of *H. niger* were able to take up 99 % of the atropine added to the medium (Hashimoto & Yamada 1983); and the accumulation of atropine by cells of *Datura* spp was inhibited by 2,4-dinitrophenol and sodium azide, and moreover, this uptake was specific to the indigenous alkaloids (Elze & Teuscher 1967). The active transport mechanism indicated by these results is probably similar to the highly specific uptake systems described for indole alkaloids by Deus-Neumann & Zenk in *Catharanthus roseus* (1984b) and *Fumaria capreolata* (1986), which were susceptible to metabolic inhibitors, substrate saturation, pH and temperature.

The gradient between the external and the vacuolar pH is likely to affect the accumulation of exogenous alkaloids by cells of *H. muticus* whether they are taken up actively or by the ion trapping mechanism. Also since a low external pH can cause the release of endogenous anthocyanins from these cells, it is conceivable that it may cause the release of endogenous tropane alkaloids from cells of *H. muticus*. However, this treatment may not cause the release of alkaloids if, as suggested, they become bound to other substances in the vacuole. The way to overcome this could be to grow the cells continuously in a medium of reduced pH, or with an increased vacuolar pH, so that the newly synthesized alkaloids pass from the cytoplasm to the culture medium without entering the vacuole. Of course it may be impossible to impose such treatments on the cells without killing them or affecting alkaloid biosynthesis.

The experiments described in Section 3.9 only identified which treatment, out of a range of permeabilization methods, may prove able to fulfil the requirements of inducing product release from immobilized cell cultures, without cell damage. More work is necessary to determine if, with the modifications suggested, the potential value of low pH treatment can be realised.

Details of the aspects which require further invesigation have been set out in the preceding sections of this chapter. Here the principle points are picked out, and objectives for future work are outlined.

The results have shown a need to identify and/or to further characterize the effects of the following treatments:

immobilization
added precursors
nutrient limitation
growth substances added to the medium

It would be useful to define these effects both on the production of alkaloids over time, and on their final yield from the cells, in order to determine the way in which they influence alkaloid metabolism. An examination of the differences between high and low producing culture lines, in their response to these treatments, may show which factors are most important in controlling the alkaloid yield from cultures of *H. muticus*.

An important part of future work will be to study more closely the biosynthesis of the tropane alkaloids. This objective can be approached by investigating the fate of radioactively labelled alkaloid precursors. Such experiments could i) provide information about changes in the activity of the biosynthetic pathway over time; ii) show if other metabolic pathways compete for the precursors; iii) identify the limiting stages of the pathway; iv) determine whether the alkaloids are subject to turnover, and the rate at which this occurs.

As discussed above, previous results (Table 4.1) have shown that root cultures generally produce more alkaloids than unorganized cell cultures, while this study found no relationship between the amount of organogenesis occurring in cultures and their atropine yield. In order to show conclusively whether root formation does affect atropine production in cultures of *H. muticus*, the roots and the unorganized cells could be analysed separately to find where the alkaloids are concentrated. This work could be carried out with a culture line which produces large numbers of roots, and one which form few, or no roots, to find out if there are any differences in the control of alkaloid production in organized and unorganized cultures of *H. muticus*.

Hall & Yeoman (1986) found that treatments which increased the anthocyanin content of cultures of *Catharanthus roseus* also increased the pigmented (productive) population; and this factor was identified as a major limitation to the anthocyanin yield from these cultures. If histological techniques could be developed to detect the small amounts of alkaloid present in cultured cells of *H. muticus*, the productive population could be determined in the different culture lines, and after different treatments, to provide more information about the control of secondary metabolism in this species.

The investigation of the aspects described above may provide information about the control of tropane alkaloid production *in vitro*. It will also be necessary to persue certain aspects that apply specifically to the production of secondary metabolites by immobilized cell cultures. These are; the development of a permeabilization method which causes the release of alkaloids from the cells without substantial loss of their viability or productive ability, and the determination of conditions which permit the long term maintenance of immobilized cells in a viable, highly productive state.

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10. THE RELATIONSHIP BETWEEN TROPANE ALKALOID PRODUCTION AND STRUCTURAL DIFFERENTIATION IN PLANT CELL CULTURES OF ATROPA BELLADONNA AND HYOSCYAMUS MUTICUS

M.A. Collinge & M.M. Yeoman

Botany Department,
University of Edinburgh,
King's Buildings, Mayfield Road,
Edinburgh. EH9 3JH. U.K.

INTRODUCTION

The tropane alkaloids atropine (L-hyoscyamine) and scopolamine are widely used anticholinergic drugs which are obtained from plants. Atropa belladonna (Deadly Nightshade) is the species most commonly cultivated for production of atropine. The dried leaves contain 0.3-0.5%, and the roots 0.4-0.6% of tropane alkaloids. The major alkaloid is L-hyoscyamine. Other alkaloids including scopolamine, and the N-oxide of hyoscyamine and scopolamine are present in varying proportions in different tissues, and at different stages of the growth cycle (James, 1950; Phillipson & Handa, 1975, 1976). A richer source of hyoscyamine is Hyoscyamus muticus (Egyptian Henbane) which is gathered from the wild in Egypt, and is cultivated in Nigeria and on a small scale in northern India. The dried leaves and flowering tops contain 0.35-1.39% of total alkaloid, 90% of which is hyoscyamine (Morton, 1977). The immediate precursors of hyoscyamine are tropine and tropic acid, which are derived from ornithine and phenylalanine respectively. Tropane alkaloids are secondary metabolites and as such are associated with differentiation.

In this study cell cultures of A. belladonna and H. muticus are being used to study the factors controlling tropane alkaloid formation and accumulation and to develop a procedure to increase the amount of alkaloid produced, which could feasibly be used for the production of hyoscyamine in vitro, on an industrial scale.

Previous work has shown that generally the more highly aggregated slow growing and structurally differentiated cultures produce higher levels of secondary metabolites (Yeoman et al., 1980; Yeoman et al., 1982; Lindsey & Yeoman, 1983a). Immobilisation of plant cells results in slow growth, higher cell-cell contact, and the establishment of chemical and physical gradients; and yet allows manipulation of the culture environment (Lindsey & Yeoman, 1983b, 1984). Cells are immobilised in polyurethane foam by a simple procedure (Lindsey et al., 1983). Polyurethane foam blocks (1 cm^3) are added to a newly subcultured suspension culture. During the following 14 to 21 days, the cells are entrapped and grow in the pores until the blocks are densely packed. The pattern of growth during the immobilisation process is illustrated in Fig. 1.

METHODS OF ANALYSIS

The system of analysis used must be able to measure accurately small amounts of alkaloids, and to separate the different alkaloids and their precursors. High performance liquid chromatography (HPLC) best satisfies these requirements. The extraction method and HPLC system described here have been derived from that of Baumann (personal communication).

Extraction Procedure

The tissue was macerated with 5% ammonia in methanol and left to stand overnight. The filtered extract was concentrated under reduced pressure and taken up in 15ml 0.1N hydrochloric acid. The acid extract was filtered and made alkaline with a buffer composed of 10ml 0.2M ammonium chloride and 9.6ml 25% ammonium hydroxide; made up to 20ml with water, and added to the top of an "Extrelut" column (Merck). After 20 minutes, the column was eluted with 40ml chloroform. The chloroform was evaporated to dryness, and the residue stored in methanol at approximately 5°C. The extracts were resuspended in the HPLC mobile phase before analysis.

Chromatographic Analysis

HPLC analysis was performed with a Gilson 302 liquid chromatograph, fitted with a Gilson u.v. detector, and a Shimadzu Chromatopac C-RIB data processor. 20µl samples were separated at ambient temperature on a Spherisorb S5 Octyl 25cm x 46mm column (Phase Separations Ltd.). The column was eluted with an isocratic mobile phase consisting of 22.5% acetonitrile and a buffer containing 50mM potassium dihydrogen orthophosphate adjusted to pH 3.0 with orthophosphoric acid. Detection was a 254nm.

Typical HPLC traces are shown in Fig. 2. Standards of the three most abundant alkaloids; hyoscyamine, scopolamine and hyoscyamine-N-oxide are clearly resolved by this system, as shown in Fig. 2a. Figs. 2b and c show the separation of hyoscyamine from the other components of

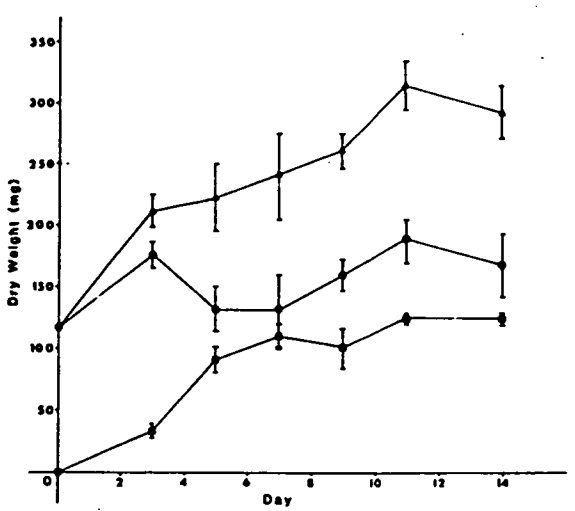


Figure 1. The changes in the dry weight of total (triangles), suspended (circles), and immobilised (squares) cells of *H. muticus* during immobilisation. Each value is the mean of three replicates ± S.E.

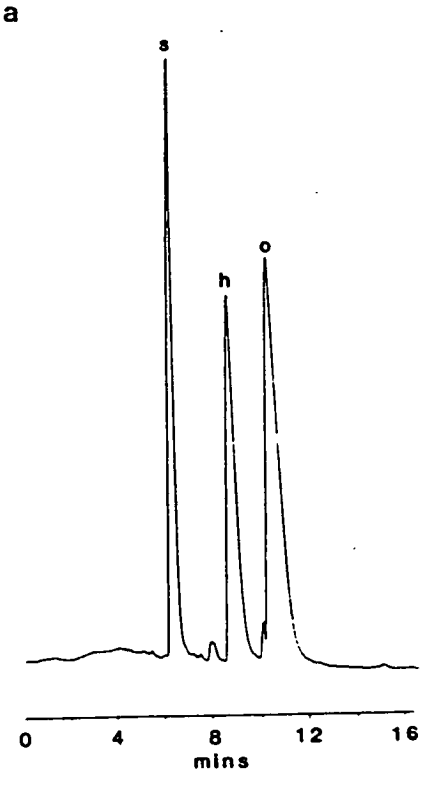
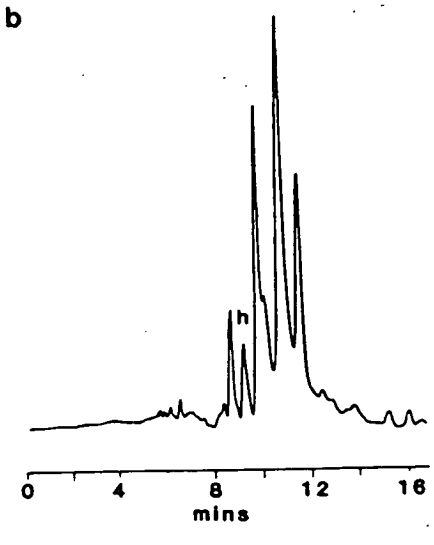
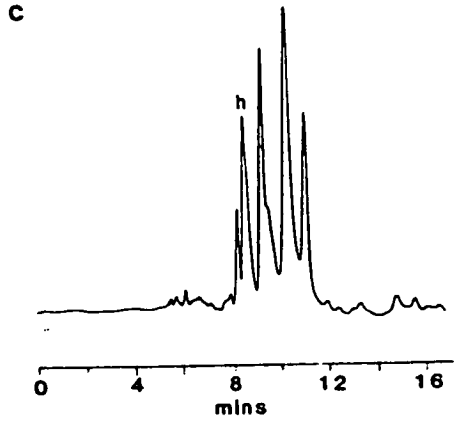


Figure 2a. Typical HPLC separation of standard drugs: scopolamine(s), hyoscyamine (h), and hyoscyamine-N-oxide (o).

Figure 2b. HPLC profile of an extract from Atropa callus containing hyoscyamine (h).

Figure 2c. HPLC profile of the same Atropa callus extract after the addition of an internal standard of hyoscyamine.





a tissue culture extract and its identification by the use of an internal standard. The callus extract alone was analysed first (Fig. 2b). Then an internal standard of hyoscyamine was added to the extract, resulting in the trace shown in Fig. 2c, where the area of the hyoscyamine peak has increased. The identity of the hyoscyamine peak in extracts has also been confirmed by spectral analysis of the peak using the diode array detector of a Hewlett Packard 1090 liquid chromatograph, and by thin layer chromatography.

ALKALOID PRODUCTION IN DIFFERENTIATED CULTURES

Early work with A. belladonna showed that alkaloids were only accumulated in excised root and root callus cultures (West & Mika, 1957) or in tissue cultures in which roots had differentiated (Thomas & Street, 1970). However, alkaloids have been produced in callus cultures (Khanna et al. 1976; Eapen et al. 1978a,b; Sharma & Khanna, 1982), and in suspension cultures (Lindsey & Yeoman, 1983); but higher levels were always found in slow growing or highly differentiated cultures. Abnormal alkaloid patterns in callus, but not in differentiated shoot buds, were noted by Eapen et al. (1978a, b).

By inducing morphogenesis in immobilised cultures, yields may be improved, and the alkaloid profile may be closer to that in vivo. Reports in the literature (Konar et al. 1972); Thomas & Street, 1972; Gosch et al. 1975) have indicated that the induction of differentiation in A. belladonna is readily achieved, but there are no similar studies with H. muticus. In this investigation experiments have been performed to find conditions which induce morphogenesis, and to characterise the types of structures formed.

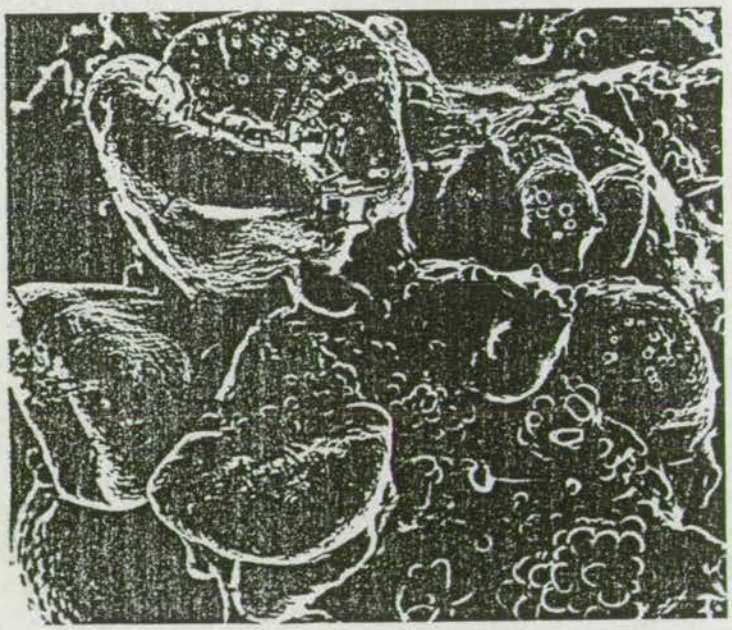
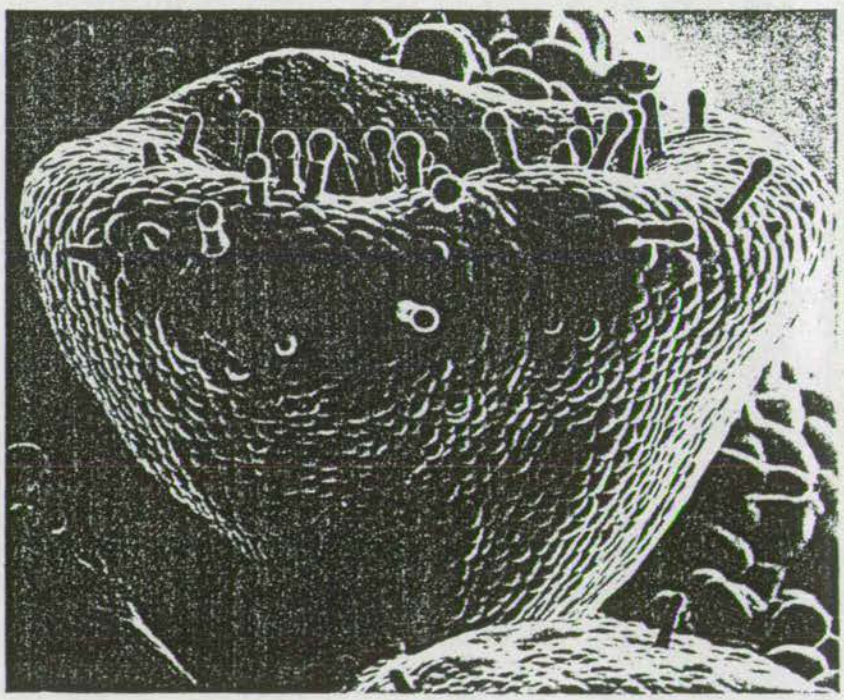


Figure 3a. Scanning electron micrograph of cryopreserved H. muticus callus showing a mass of callus in which a number of shoots have developed.

Organogenesis can be induced in Murashige & Skoog medium (Flow Laboratories). Cells are grown first in a primary induction medium which contains 1.0 $mg.ml^{-1}$ para-chlorophenoxyacetic acid, 0.5$mg.ml^{-1}$ 2,4-dichlorophenoxyacetic acid and 0.1$mg.l^{-1}$ kinetin. The secondary induction medium, in which organogenesis occurs contains only 0.1$mg.ml^{-1}$ kinetin. *H. muticus* usually exhibits more differentiation than *A. belladonna* under these conditions. Both suspension and immobilised cell cultures form roots and undeveloped primorida. In callus both roots and shoots were formed. Scanning electron micrographs of cryopreserved developing shoots in *H. muticus* callus are shown in Fig. 3.

At present, the effect of this structural differentiation on the pattern and extent of alkaloid production is being determined, and compared with that of cultures which do not display structural differentiation. In this way, some understanding of the effect of differentiation, and the form it must take to achieve, or approach the alkaloid metabolism of the intact plant will be gained. If a positive correlation is obtained, this induction of structural differentiation could be incorporated into the final *in vitro* alkaloid production system together with other treatments such as nutrient, limitation or precursor feeding in order to increase the yield of hyoscyamine from the cultured cells.

Figure 3b. Scanning electron micrograph of cryopreserved *H. muticus* callus showing a single developing shoot at high magnification.



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Ways and means to plant secondary metabolites

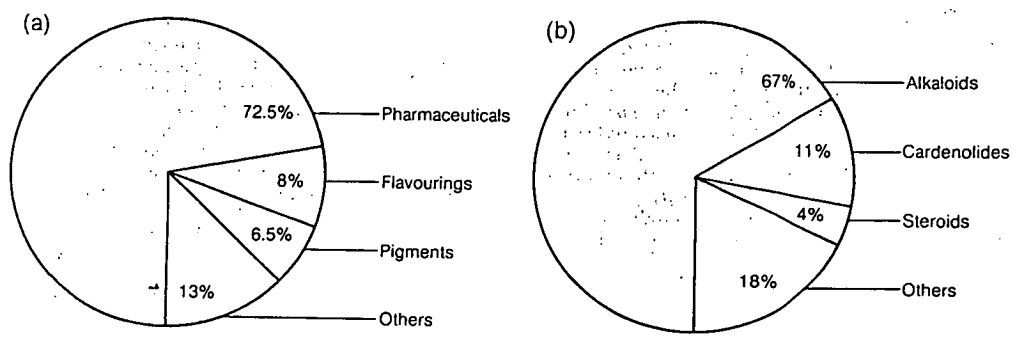
Considerable efforts have been made over the years to produce useful secondary products by plant tissue culture. Figure 1 indicates the relative emphasis placed on the production of different kinds of products, as shown by the proportion of papers describing work on each product type at the 6th International Congress of the International Association for Plant Tissue Culture, held at the University of Minnesota in August 1986. The emphasis on pharmaceuticals probably stems from their being of high value, but required only in relatively small quantities; and from each required drug being a discrete, single compound. Flavours, food additives and perfumes, on the other hand, are made in large amounts, for less unit cost; also, a number of these products are made up of complex mixtures of compounds and are thus more difficult to produce and quantify in tissue culture.

The principal problem is the low yield of secondary products from most plant cell cultures. There are examples of cultures which equal or, more rarely, exceed the levels of product found *in vivo*, but further increases in yield will be necessary before natural product production by plant cultures can be economically viable. This was the problem to which most papers on secondary metabolism were directed.

Plant cell cultures which accumulate high levels of secondary metabolites can be established by identifying high yielding lines and developing optimum culture conditions. Both selection and screening can be used to identify high yielding cell lines. Lines which overproduce the amino acid precursor to a desired product can often be selected for as they are resistant to toxic analogues of the amino acid. Failing this, high yielding lines can be distinguished by screening, the ease of which depends on the simplicity and sensitivity with which the metabolite can be detected and determined. If the product is a pigment (e.g. shikonin) or detectable by its fluorescence (e.g. indole alkaloids), single cells or small aggregates can be screened. Where the product cannot be detected visually, single cells must be grown to produce large aggregates. If an immunological method of analysis is available, relatively small cell aggregates can be screened quickly and in large numbers. When this is not possible, or suitable, extracts from larger aggregates must be analysed using less sensitive and more time-consuming methods, usually GLC or HPLC.

At the IAPTC Congress, Frankhauser *et al.* reported the use of an enzyme immunoassay for scopolamine to screen for high yielding clones of *Hyoscyamus muticus*, while Cocking, Galbraith *et al.* and Lucretti *et al.* spoke on the adaption

Fig. 1



(a) The proportion of reports at the IAPTC meeting that dealt with secondary products of each type (classified by their application). (b) The proportion of reports for each product type, of those that have application as pharmaceuticals.

of the fluorescence activated cell sorting (FACS) technique for sorting plant cells. Although FACS has been used for some years in sorting microbial and animal cells, there have been difficulties in applying it to sort larger, more irregular, plant cells. There were no reports of any efforts being made to use cell sorting machines for the direct selection of high yielding lines, although Tabata and Takahashi *et al.* outlined their plan to use FACS to select fusion products between protoplasts of slow-growing, high-yielding cell lines and fast-growing, low-yielding lines of *Lithospermum erythrorhizon*, for the production of shikonin.

Cultural conditions

In general, the conditions which promote secondary metabolism in tissue cultures are those that allow limited growth. Thus a two-stage culture system is often suggested as the best way to produce natural products: the first stage allows fast growth and biomass accumulation, then conditions are changed to support slow, or no, growth and to favour secondary metabolite synthesis and accumulation. The factors which are manipulated include light, temperature, culture vessel agitation and aeration, but the most influential factor is the composition of the culture medium.

Composition of the medium

Both the quality and concentration of growth regulators, particularly of auxins, affect secondary metabolism. Usually there is a reduction in concentration and/or a change to a weaker auxin between the growth and production medium. The effect of cytokinins on secondary metabolism is variable.

Previously there has been little work on the effect of other growth regulators. However, at this meeting Morimoto *et al.* reported that treatment of cultures of *Coptis japonica* with gibberellic acid decreased the starch content of the cells and increased their berberine content. Ohlssen *et al.* described a light-sensitive strain of *Digitalis lanata* which accumulated cardenolides only in the dark: gibberellic acid treatment resulted in a four-fold increase in cardenolide yield from these cells. Cell lines which were not light-sensitive showed a negative or neutral response to this treatment.

Secondary metabolism can also be promoted by altering the concentration of the macro- and micro-nutrients in the medium. Limitation of phosphate is most frequently successful in increasing product yield, but reduction of the nitrogen concentration, or a change in the nitrogen source, has also proved effective.

Stress

Many secondary metabolites are thought to have a role in the plant's defence against viral, microbial or herbivore attack and their synthesis is induced under conditions of stress.

Increasing the concentration of sucrose in the medium has been used to increase yields. It is thought that this may be due not to a relief of carbon limitation, but to stress caused by the increased osmotic potential of the medium. In support of this idea is the report of Frischknecht *et al.*, who showed that increasing the osmotic potential of the medium by the addition of mannitol, which is not metabolized, stimulated indole alkaloid production by *Catharanthus roseus* cultures by a factor of nine.

Smart *et al.* investigated the effect of osmotic stress on indole alkaloid production by cells of *Catharanthus roseus*; the major factor causing stress was abscisic acid, which was added to the medium at high concentration. Other means of causing stress included the use of actinomycin-D, nigeran or sodium vanadate to induce stress metabolites of *Vigna angularis*, and induction of stress metabolites of *Solanum melongena* by nigeran or autoclaved RNase A (Ohta *et al.*). A stress metabolite of *Glycyrrhiza echinata*, echinatin, was produced in larger amounts, and for a longer duration, when suspended cells were immobilized in calcium alginate than when simply transferred to fresh medium. However, the addition of alginate to the culture medium had the same effect as immobilization (Ayabe *et al.*). Similarly, when Haldiman and Brodelius immobilized *Coffea arabica* cells in order to increase production of methylxanthins, they found that levels were increased only when alginate was used as the immobilization matrix. The pattern of methylxanthins in immobilized cells was different to that in freely suspended cells and in the intact plant. Thus it appears that alginate too can be used to impose stress on cells.

Fungal elicitors

The group of chemicals used for inducing stress which are now attracting most attention are the fungal elicitors. These substances trigger the production of phytoalexins, plant secondary metabolites which contribute to the plant's defence against disease. In tissue culture systems, elicitors have been used to stimulate, or induce, the production of known phytoalexins [e.g. sanguinarine in *Papaver* spp. (Constabel *et al.*, Cline and Coscia), glyceollin isomers in *Glycine max* (Brodelius *et al.*)] and of metabolites which have no known, or proven, role in defence [e.g. anthraquinones in *Rubiaceae* spp. (Verpoorter *et al.*), berberine in *Thalictrum rugosum* (Brodelius *et al.*) and indole alkaloids in *Catharanthus roseus* (Talluvi *et al.*)]

There are a number of advantages in using elicitors. Their preparation is quite simple, being anything from an autoclaved mycelial suspension or crude culture filtrate to proteinaceous or polysaccharide extracts. Large increases in yield can be obtained in very short periods of time – hours, rather than the usual days. Cells can be re-elicited, and there is no need for a two-stage system. The use of elicitors will, no doubt, be rapidly extended to other plant species and other products. Their large and rapid effect on cultured cells will be particularly useful in studies on secondary metabolic pathways and their regulation.

However, there will no doubt be limits to their use, as they appear to be able to trigger only certain secondary metabolites in each plant species examined. The *Papaver somniferum* cultures of Constabel *et al.* and Cline and Coscia accumulated sanguinarine but no morphinan alkaloids upon elicitation. Similarly, levels of some indole alkaloids were

increased in elicited cultures of *Catharanthus roseus*, but these did not include the antineoplastic agents, vindoline and the bis-indole alkaloids.

Differentiated cultures

Despite the various methods used to manipulate the medium, plant cells still fail to produce more than trace amounts of certain metabolites while in disorganized suspension culture. There is evidence that in addition to slow growth, close cell–cell contact, aggregation and differentiation are also necessary for the expression of secondary metabolism. These conditions can be achieved when cells are immobilized, usually within a polymeric matrix. Lindsey and Yeoman reported enhanced yields of the pungent principle capsaicin from *Capsicum frutescens* cells immobilized in polyurethane foam.

With some other plant species, even organized cultures do not accumulate products to any marked extent, and morphological differentiation appears to be necessary to enhance production. Examples include the production of cardenolides by embryogenic cultures of *Digitalis lanata* (Luckner *et al.*), by organogenic cultures of *Asclepias currasavica*, *Calotropis gigantea* and *Thevetia peruviana* (Pramanik *et al.*), and the production of tropane alkaloids in root-forming cultures of various members of the Solanaceae. Yamada *et al.* reported the production of tropane alkaloids by root cultures of a number of species; for instance, yields equivalent to, or greater than, those in the intact plant were obtained using a culture line of *Hyoscyamus niger*. However a two-stage culture system was required, and the roots grew comparatively slowly even in the growth medium.

A more promising development is the use of root cultures which have been transformed by *Agrobacterium rhizogenes*, the bacterium which causes 'hairy root' disease. Such root cultures have high growth rates, and accumulate secondary products in the same proportions as the parent plants. Growth rate and yield appear to be stable through a number of culture passages. So far this technique has only been applied to the production of tropane alkaloids by 'hairy root' cultures of *Hyoscyamus*, *Scopolia*, *Atropa* and *Datura* spp. (Flores *et al.*, Shimomura *et al.*). In future it may be extended to increase the accumulation of other useful products, but it will probably be limited to those products which are synthesized chiefly in the roots of the intact plant.

Regulation of pathways

Each of the methods already described for increasing or inducing natural product accumulation seems to be appropriate only to a limited number of systems, and they are largely empirical. It is unlikely that a single, simple method will be found which can switch on all the useful secondary pathways. Tissue culture yields of many of the really useful and valuable products still need to be improved. In order to realise the biotechnological potential of plant secondary metabolism, much more precise research must be undertaken into the pathways, and their regulation at the molecular level. Manipulation of the expression of secondary pathways in plant cell cultures may then be undertaken to (a) increase yields, (b) perhaps also cause release of the product into the medium, and (c) direct biotransformations or the synthesis of novel compounds by modifications of existing pathways.

There were reports at the Congress on the study of pathway regulation (Brodelius *et al.*, De-Eknamkul *et al.*, Endo *et al.*, Hall *et al.*, Ruetter, and Zenk *et al.*), but they represented only a small proportion of the papers presented on secondary metabolism. Studies of this type are on the increase however, and when their results are coupled with the advances already made, and with advances in plant molecular biology and genetic manipulation, economic *in vitro* commercial production of more natural products will become possible.

MARGARET COLLINGE

Department of Botany, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, UK.